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MENINGEAL CELLS CONTRIBUTE TO CORTICAL NEUROGENESIS IN POSTNATAL BRAIN

IN *CO-TUTELLE DE THÉSE* WITH THE UNIVERSITY OF LEUVEN
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ABBREVIATIONS

VZ	Ventricular zone
RGC	Radial glial cell
SVZ	Subventricular zone
NSC	Neural stem cell
BP	Basal progenitor
MZ	Marginal zone
PP	Preplate
SP	Subplate
CP	Cortical plate
OB	Olfactory bulb
RMS	Rostral migratory stream
SGZ	Subgranular zone
DG	Dentate gyrus
CNS	Central nervous system
BrdU	Bromodeoxyuridine
GCL	Granular cell layer
Ascl1	Achaete-scute homolog 1
Brn2	POU domain
Myt1l	Myelin Transcription Factor 1-Like
PAX6	Paired box protein 6
Sox2	SRY (sex determining region Y)-box 2
NG2	Neural/glial antigen 2
DCX	Doublecortin
GFAP	Glial fibrillary acidic protein
GAD65/67	Glutamic acid decarboxylase 65-67
GABA	Gamma-Aminobutyric acid(γ-Aminobutyric acid)
Tuj1	Neuron-specific class III beta-tubulin
CSF	Cerebrospinal fluid
SC	Spinal cord
E1…E18	Embryonic day 1…18
SDF1	Stromal cell-derived factor 1
CXCL-12	C-X-C motif chemokine 12
CXCR4	C-X-C motif receptor 4
Shh	Sonic hedgehog
RA	Retinoic acid
BMP7	Bone morphogenetic protein 7
Wnt3	Wingless-Type MMTV Integration Site Family, Member 3
ECM	Extracellular matrix
bFGF	Basic fibroblast growth factor
CX	Connexin
FGF2	Fibroblast growth factor 2

EGF	Epidermal growth factor
FGFR1	Fibroblast growth factor receptor 1
LeSC	Leptomeningeal stem/ progenitor cells
MAP2	Microtubule associated protein 2
CD90	Cluster of differentiation 90
PDGFRα	Platelet derived growth factor receptor A
GalC	Galactocerebroside
LV	Lentiviral vector
GFP	Green fluorescent protein
NSPC	Neural stem precursor cell
Hq mice	Harlequin mice
EdU	5-ethynyl-2'-deoxyuridine
WT	Wild-type
Tg	Transgenic
Wnt1	Wingless-Type MMTV Integration Site Family, Member 1
PDGFRβ	Platelet derived growth factor receptor B
GLAST	Glutamate aspartate transporter
YFP	Yellow fluorescent protein
P0…P30	Postnatal day 0…30
CFSE	Carboxyfluorescein succinimidyl ester
BSA	Bovine albumine serum
FBS	Fetal bovine serum
DiI	1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate
ACSF	Artificial cerebrospinal fluid
PFA	Paraformaldehyde
DAPI	4',6-Diamidino-2-Phenylindole Dihydrochloride
H&E	Hematoxilin and eosin
RFP	Red fluorescent protein
NeuN	Neuronal nuclei
Satb2	Special AT rich binding protein 2
HuC/D	Human neuronal protein C/D
Blbp	Brain lipid binding protein
Iba1	Ionized calcium-binding adapter molecule 1
CFP	Cyan fluorescent protein

SUMMARY

Neurogenesis continues throughout life in mammalian brain (Eriksson et al., 1998; Gage, 2000) in two germinal niches: the subventricular zone and subgerminal zone in the dentate gyrus of the hippocampus (Gage and Temple, 2013). Radial glial cells (Kriegstein and Alvarez-Buylla, 2009) are the neural stem cells that, during embryonic and postnatal development, give rise to various cell types including neuroblasts, neurons, oligodendrocytes, astrocytes and ependymal cells (Kriegstein and Alvarez-Buylla, 2009). In adult mice, newly formed neuroblasts migrate through the rostral migratory stream to the olfactory bulb, where they continually replace local interneurons (Imayoshi et al., 2008).

Apart from these well-established neural stem niches, the existence of ectopic neural stem cell niches has been reported following injury (Pluchino et al., 2010), as well as in selected physiological conditions in the retina, cerebellum and olfactory bulb (Menezes et al., 1995; Ponti et al., 2008; Tropepe et al., 2000).

Interestingly, several independent groups have recently identified a novel role for meninges as a potential niche harbouring endogenous stem cells with neural differentiation potential in the adult brain (Bifari et al., 2009, 2015; Decimo et al., 2011; Nakagomi et al., 2011, 2012; Petricevic et al., 2011). Surprisingly, meningeal neural precursors are able to differentiate both *in vitro* and, after transplantation *in vivo*, into neurons with extremely high efficiency (Bifari et al., 2009; Decimo et al., 2011). Moreover, these cells can be activated by CNS parenchymal injuries, undergoing an extensive expansion of stem cells and progenitors (Nakagomi et al., 2012). Meningeal neural precursors contribute to neural parenchymal reaction after spinal cord injury, migrating to the perilesioned area, while expressing the same markers (nestin and DCX) that are transiently expressed by neural precursors within classic neurogenic niches (Decimo et al., 2011).

The finding of this new cell population in the meninges, with stem cell features, provides new insights into the complexity of the parenchymal reaction to a traumatic injury and suggests a potential role for meningeal progenitor cells in the maintainance of brain homeostasis. However, the possible contribution of meningeal neural precursors to neurogenesis in physiological conditions has not previously been

investigated. During the course of my studies, I explored the hypothesis that meningeal cells may contribute to neurogenesis *in vivo*.

We were able to specifically tag meningeal cells in P0 pups and track them during time, combining injection of cell tracers in the meningeal subarachnoid space and transgenic mouse lines. We found that neurogenic meningeal cells migrate from their location outside the brain parenchyma, along the meningeal substructures, to the retrosplenial and visual motor cortices during the neonatal period. Subsequently, meningeal-derived cells differentiate into cortical neurons that are electrophysiologically functional, integrated in the existing network and responsive to pharmacological stimuli. In addition we found that these meningeal neurogenic cells belongs to the perivascular PDGFRß$^{+}$ lineage and are mainly additive to the well-characterized neurogenic parenchymal radial glia. Although the developmental origin of these cells still has to be elucidated, our preliminary data indicate a possible neural crest-derivation.

Thus, a reservoir of embryonic derived progenitors residing in the meninges contributes to postnatal cortical neurogenesis. These cells may have a role as endogenous stem cell pool that can be exploited in regenerative medicine for neurological diseases.

CHAPTER 1

INTRODUCTION

1.1 - Let's talk about neurogenesis

Since the beginning of the XX century, research in adult neurogenesis is a trending and challenging topic, and new knowledge about the mechanisms underlying this phenomenon is added every day. Nonetheless, it's a controversial one. In the very beginning of the history of neurogenesis research, around the 1960s, Joseph Altman's work claiming the possibility of neurogenesis in the adult rat (Altman, 1962) was basically overlooked by the scientific community. Scientists recovered interest in this field only in the 1990s, when new data confirmed the Altman's hypothesis and new engaging studies about adult neurogenesis (Reynolds and Weiss, 1992; Gage et al., 1995a; Eriksson et al., 1998; Doetsch et al., 1999; Alvarez-Buylla et al., 2002; Gage and Temple, 2013) opened the field to the intriguing idea that these newborn cells could be use to cure neurodegenerative diseases (Bonnamain et al., 2012; Xu et al., 2011).

In the last 20 years, big efforts were made to improve the existing knowledge about adult neurogenesis, but the more the studies proceed, the more researchers underwent into a negative-pessimistic phase, with a even more clear realization about the difficulties of using these cells for cellular therapies (Cattaneo and Bonfanti, 2014; Lois and Kelsch, 2014; Peretto and Bonfanti, 2014), especially for cell replacement therapies.

In this introductory chapter, I will summarize the milestones in adult neurogenesis research, highlighting the important steps and techniques used to investigate this phenomenon. After a brief analysis of the mechanisms underlying embryonic and adult neurogenesis, I will focus on ectopic neurogenic niches, in particular on new interesting works about meninges and their potential role as neurogenic niche in adult brain.

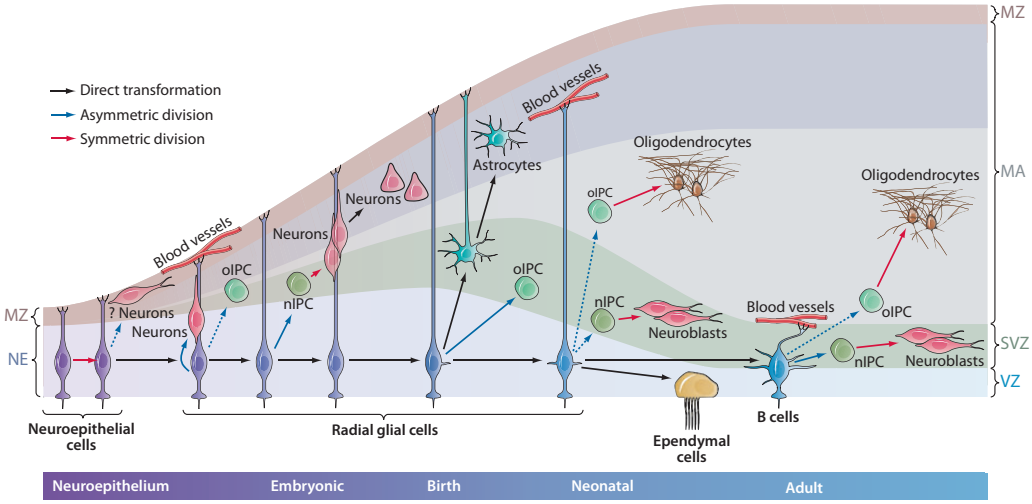

Figure 1.1. Representative scheme of neurogenic processes during development and adulthood.

As the brain epithelium thickens during development, neuroepithelial cells elongate and convert into radial glial cells (RGCs). RGCs divide asymmetrically to generate neurons directly or indirectly through neurogenic intermediate progenitor cells (nIPCs). Oligodendrocytes are also derived from RGCs through intermediate progenitor cells that generate oligodendrocytes intermediate progenitor cells (oIPCs). A subpopulation of RGCs retains apical contact and continue functioning as NSCs in the neonate. These neonatal RGCs continue to generate neurons and oligodendrocytes through nIPCs and oIPCS; some convert into ependymal cells, whereas others convert into adult SVZ astrocytes (type B cells) that continue to function as NSCs in the adult. B cells continue to generate neurons and oligodendrocytes through (n and o) IPCs. Modified from Kriegstein and Alvarez-Buylla, 2009.

1.1.1 - Brain and neurogenesis studies: a new start

The study of brain structure and development was considered a mysterious and fascinating field since the beginning of 1900. One of the first detailed description of human brain areas was provided by the german anatomist Korbinian Brodmann, who was able to classify neurons in their cyto-architectural organization using the Nissl method of cell staining (Brodmann, 1909). His maps of cortical areas in human, monkeys and other mammals permitted the division of the brain in "areas" depending on the neuronal organization, and this description was implemented in 1925 by the work of other two german scientists (Economo and Koskinas, 1925). By observation of clinical cases and appropriate neurophysiological experiments, it was possible to define the location of association cortical areas of higher order functions, as the localization of Broca's speech and language area close to Brodmann areas 44 and 45.

Immunohistochemistry and cell staining methods set the basis of the work of Santiago Ramon y Cajal. Spanish pathologist and pioneer in neuroscience, he performed an intense work of cellular classification, based on investigation of the microscopic structures of the brain in Golgi staining and, in particular, of the neuronal species, illustrating the delicate arborization of the single cell (Ramón y Cajal and Azoulay, 1894). His work was extremely important to improve the general knowledge about brain cell populations: he discovered the growth cone, supported the theory of existence of dendritic spines, discovered a new type of cell, the interstitial cell of Cajal, interleaved among neurons and embedded within the smooth muscles lining the gut, serving as the generator and pacemaker of the slow waves of contraction (Finger and Stanley, 2000; Ramón y Cajal and Azoulay, 1894).

Thanks to his extremely accurate description of the central nervous system cell population, the statement, in one of his publication, that "brain is fixed, static, unable to generate new cells" (Finger and Stanley, 2000), set the first dogma about the absence of new neurons generation in the postnatal and adult phases.

It was only in the early 1960s that this statement was questioned. Indeed, in 1962 Joseph Altman described that, in physiological conditions and following a brain trauma, new glial cells were found associated with the lesioned area (Altman, 1962, 1963). He was able to describe the presence of proliferating glia by means of injection of ^{3}H-thymidine; moreover, also some neurons and neuroblasts have been showed to be labelled, suggesting the intriguingly possibility that new proliferating neurons could be present in adult rat brain. He settled a milestone in the history of neurogenesis, but his work was not immediately considered revolutionary.

Starting from this fascinating possibility, I will review the milestones that were settled in the field of neurogenesis, starting from the embryonic development to the adult neurogenesis.

1.1.1.1 - Embryonic neurogenesis

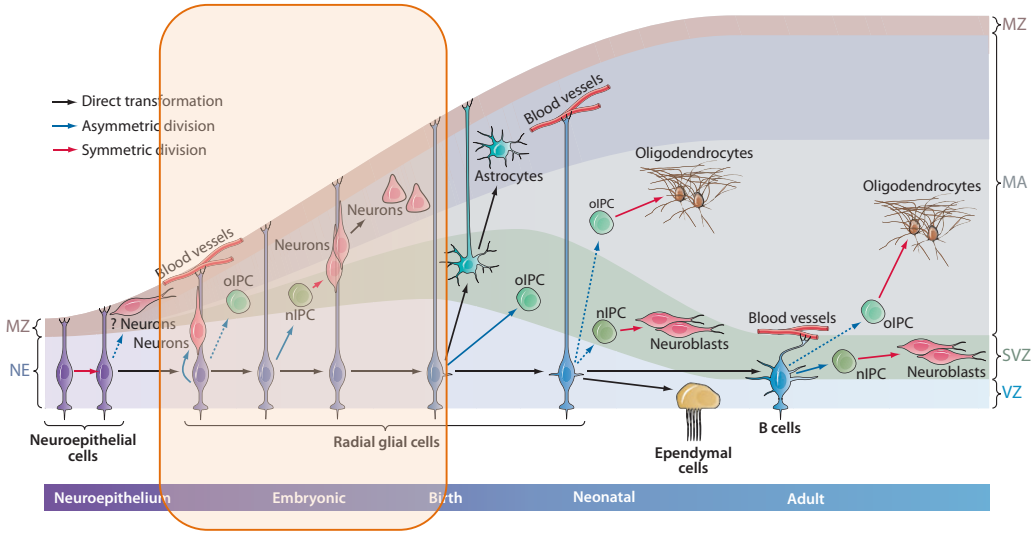

Figure 1.2. Representative scheme of neurogenic processes during embryonic development. In this chapter, we will focus on the generation of neurons during the embryonic period, highlighted in orange in the figure. Modified from Kriegstein and Alvarez-Buylla, 2009.

The generation of cortical neurons was known to be restricted only to the embryonic developmental stage. In the very early events of development, the neural tube is composed by a single layer of neuroepithelial cells (Götz and Huttner, 2005). When the neurogenic process starts, this neuroepithelium turns into a multi-layered tissue, and the specific layer that line the ventricle is referred to as the ventricular zone (VZ, Kriegstein and Götz, 2003). The newly transformed neuroepithelial cells residing in the VZ are called radial glial cells (RGCs); these cells not only display residual neuroepithelial features but, as development proceeds, they start showing astroglial hallmarks (Götz and Huttner, 2005). The region immediately adjacent to the VZ is called subventricular zone (SVZ, Doetsch et al., 1997) and it harbors the population of cells that will give rise to all the neurons and the glial cells in the brain: the neural stem cells (NSCs, Doetsch et al., 1999). Neuroepithelial cells can undergo a symmetric, proliferative division (Figure 1.3) generating two daughters neuroepithelial cells, and an asymmetric, differentiative division, where a RGC and a basal progenitor (BP) are generated. BPs can undergo symmetric, neurogenic division only, and it is in this step that new neurons are formed. RGCs, on the contrary, can still undergo an asymmetric division, where a RGC and a BP are generated.

Eventually, BPs differentiate and each generates two neurons (Huttner and Brand, 1997; Figure 1.3).

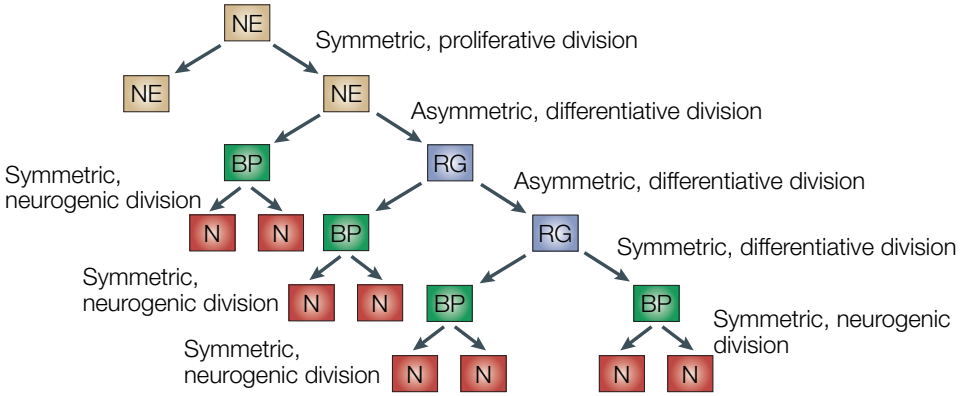

Figure 1.3. Division events that occur during neurogenic events in the SVZ.
The first event of division from a single neuro-epithelial cell (NE) gives rise to two NE-daughter cells (symmetric, proliferative division); one of those give rise to a radial glia cell (RG) and a basal progenitor (BP) with an asymmetric, differentiative division. A BP divide symmetrically and differentiate into neuron (N), while a RG can undergo an asymmetric, differentiative division (one BP and one RG) or a symmetric, differentiative division (two BPs). Modified from Götz and Huttner, 2005.

These processes are now well known and described, but big efforts were made since the beginning of the 1930 to understand the mechanism underlying embryonic neurogenesis.

The first description of the mechanism of RGCs undergoing interkinetic nuclear migration was provided by F. C. Sauer in 1935. Although RGC bodies are restricted to the VZ, they exhibit movements along the apical-basal axis within the VZ, dependent upon different stages of the cell cycle (Sauer, 1935; Figure 1.4). Indeed, a RGC body contacts the ventricular surface (apically) during mitosis (M phase), moves away from the ventricle during G1, reaches the basal VZ as it begins DNA-synthesis (S-phase), then toward the apical surface during G2 (Götz and Huttner, 2005; Noctor et al., 2004; Taverna and Huttner, 2010).

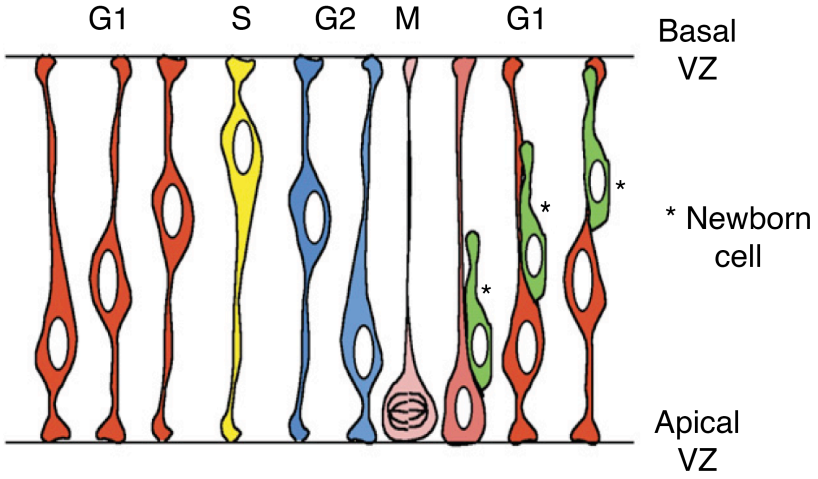

Figure 1.4. Interkinetic nuclear migration of RGCs during different stages of the cell cycle. In the G1 phase (red), RGC bodies are restricted to the apical VZ, while they move basally during the S phase (yellow). They move back apically during the mitotic division (pink), and new cells are there generated (green). Modified from Guerrier and Polleux, 2007.

In the beginning of the 60's, JB Angevine and Richard Sidman demonstrated active cell migration during the histogenesis of mouse neocortex (Angevine and Sidman, 1961). By using injections of ^{3}H-thymidine and autoradiographic studies they identified the events guiding proliferation and migration of newborn neuronal cells during the stages of cortico-histogenesis, discovering the "last-in / first-out" disposition of newly generated neurons. Indeed, this work allowed them to determine that the first generated neurons will be the one that first migrate to the "superficial-most" layer of the putative cortex, called marginal zone (MZ) in embryo and to be replaced with layer 1 in the postnatal period. The earliest born neurons form the preplate (PP) around E11.5, which later splits into the more superficial MZ and the deeply located subplate (SP) at E12.5 (Molyneaux et al., 2007). The cortical plate (CP), which will give rise to the 6-layered neocortex, develops progressively between these two layers (PP and SP), in an opposite way ("first-in / last-out") compared to the MZ. Indeed, later born neurons arriving at the CP migrate over (and thus settle medially, under the MZ) earlier born neurons (settled in the lower layers; Figure 1.5).

Regarding cortical layer formations, notable was the work of Bayer in 1990, where, together with Altman, provided an accurate description of cortical layer 1 and SP development (Bayer and Altman, 1990). With the injection of ^{3}H-thymidine in

pregnant females at different embryonic stages, to analyze the proportion of newly formed neurons at each embryonic day, they described that presumptive Cajal-Retzius cells (large horizontal cells present in the MZ of the cortex and secreting reelin) are generated mainly on E14, while SP cells on E14 and E15. An accurate description of the cortical layers formation in an evolutionary perspective was provided in 1978, from Marin-Padilla, with a detailed analysis of modifications occurring in neocortex across different species (Marin-Padilla, 1978).

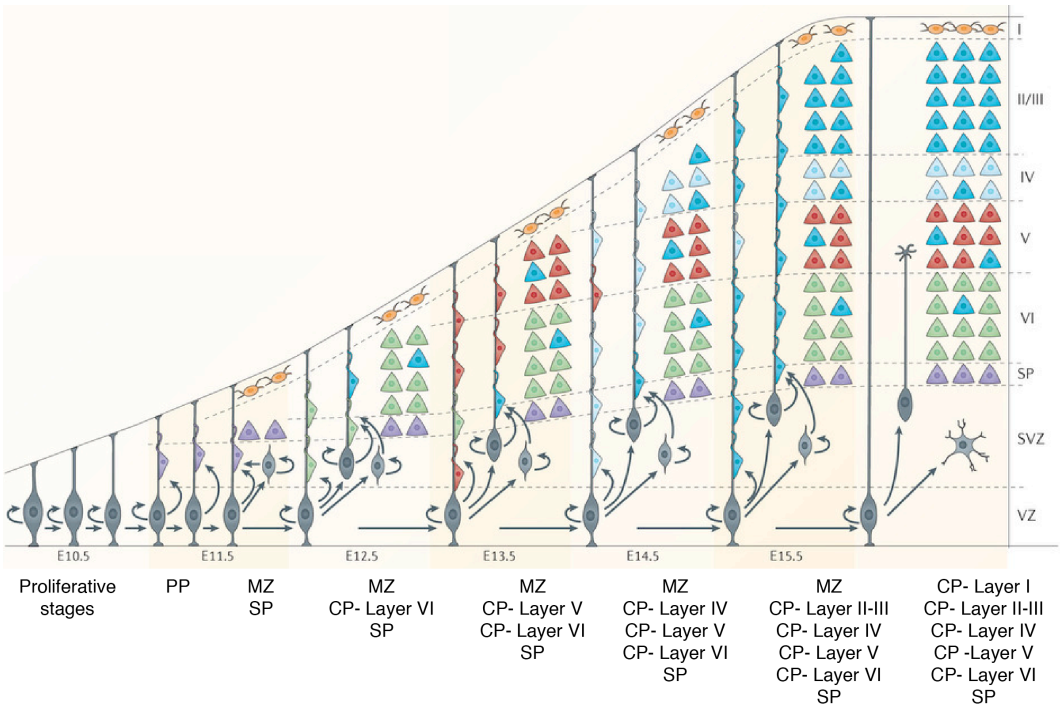

Figure 1.5. Cortical development and layer formation during embryonic development.
This scheme depicts the sequential generation of neurons and their migration to appropriate layers over the course of mouse embryonic development. Radial glia cells (RGCs, in grey) in the ventricular zone (VZ) begin to produce projection neurons around embryonic day 11.5 (E11.5). At the same time, RGCs generate intermediate progenitors (IPs, grey) and outer RG (oRG, grey), which act as transit-amplifying cells to increase neuronal production. Cajal–Retzius (CR, orange) cells primarily migrate into the marginal zone (MZ) from non-cortical locations, whereas other projection neurons are born in the neocortical VZ and/or SVZ and migrate along RG processes to reach their final laminar destinations. Modified from Greig et al., 2013.

Works from Caviness and colleagues were important in the description of the neocortical histogenesis (Caviness, 1982; Caviness and Sidman, 1973; Caviness et al., 1995), specifically in the definition of the importance of reelin in layer distribution in

the newly forming cortex. His works showed that, despite the normal generation of neuronal classes and subtypes in mouse mutant for the reelin gene (the releer mutant model), the relative positions of the principal neuronal classes of neocortex are inverted, showing anomaly of migrations and the post-migratory positions of neurons. Indeed, reelin is not only an essential factor in the control of neuronal migration and layer formation in the developing mammalian brain, but is also involved in the positioning of neurons in the developing brain, their growth, maturation, and synaptic activity in the adult brain (D'Arcangelo, 2014).

In parallel independent groups were studying the development of cerebellum, in murine models (Suginoshita, 1971), monkeys (Rakic, 1972), and rats (Raedler and Raedler, 1978).

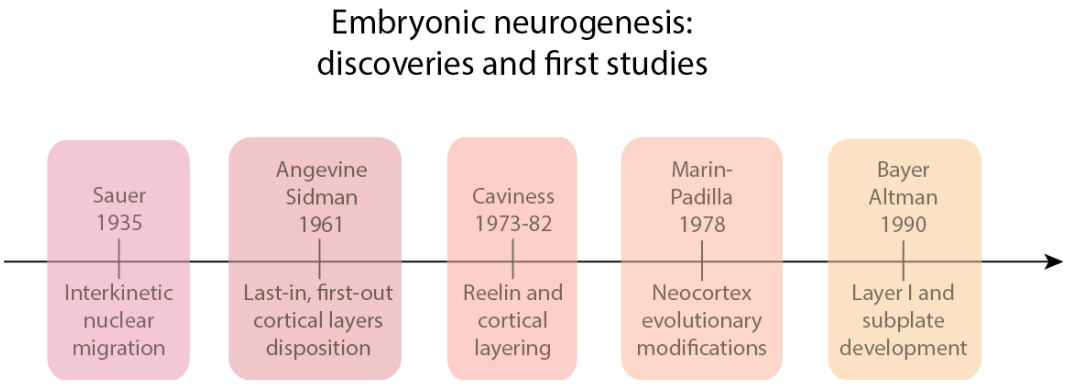

Figure 1.6. Schematic timeline of studies on embryonic neurogenesis.

This scheme depicts the timeline of some of the important studies and discovery about the embryonic neurogenesis, starting from the work of Sauer in 1935, to the work of Altman and Bayer in 1990.

1.1.1.2 - Adult neurogenesis

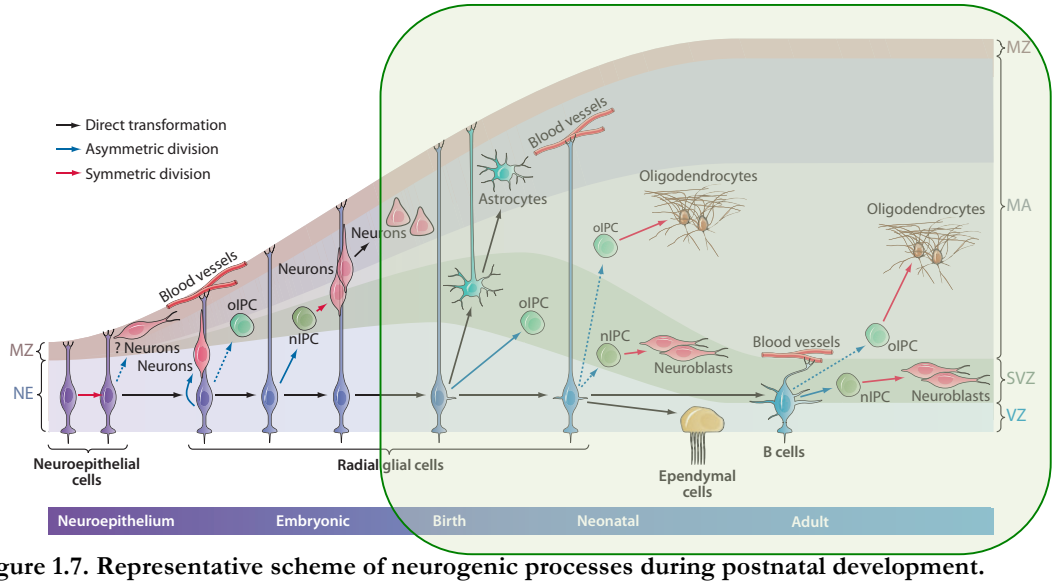

Figure 1.7. Representative scheme of neurogenic processes during postnatal development. In this chapter, we will focus on the generation of neurons during the postnatal period, highlighted in green in the figure. Modified from Kriegstein and Alvarez-Buylla, 2009.

As mentioned before, Ramon j Cayal in the early 1950s stated that adult brain is unable to generate new neurons, and it's fixed in its condition.

However, already in 1912 the first mention of new neuron production in the adult mammalian brain was provided, showing dividing cells in the walls of lateral ventricles of rats (Allen, 1912).

The already impressive works of Altman in the 1960s passed pretty unobserved, but he received feedback soon after setting what will be one of the most important discoveries in brain research. He indeed described the migration of newly formed neurons in an anterior path, leading to the most rostral part of the brain, the olfactory bulbs (OBs). He described the rostral migratory stream (RMS, Figure 1.8), one of the most important path of migration of new neurons in the adult brain (Altman, 1969). Neuronal precursors, generated in the SVZ, migrate in this anterior path, in a stream of cells to the OBs.

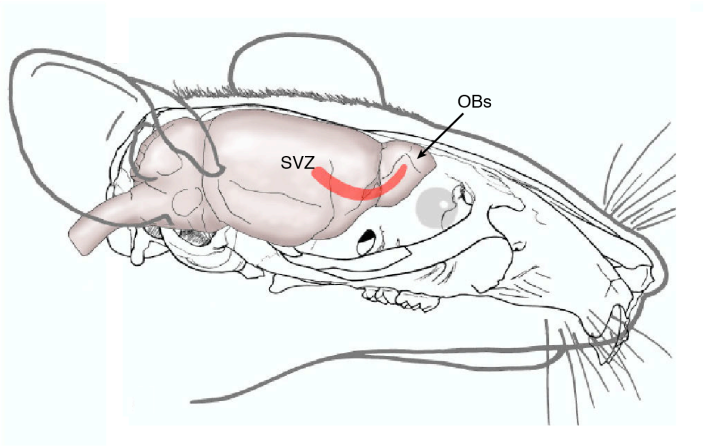

Figure 1.8. Schematic draw of the RSM localization in rodent brain.

The scheme represents the localization of the brain (pink) in the rodent skull; the RMS path of migration, from the SVZ to the OBs, is highlighted with the red line. Modified from Lennington et al., 2003.

An important contribution was given by the group of James W Hinds (Kaplan and Hinds, 1977). With the injection of ^{3}H-thymidine in 3 months old-rats, he was able to identify by electron micrographs labelled cells in the granular layers (subgranular zone, SGZ) of dentate gyrus (DG) and OB, confirming that new proliferating neurons are settled in those regions.

This important statement was confirmed by some other works that arrived immediately after, regarding volumetric and numeric differences in the postnatal brain cortex (Bayer, 1982). Indeed, the density of granule cells in the DG increases 43% between day 30 and 365, while the total volume of this region decreases (Bayer, 1982); moreover, the net number of neurons in the rodent cerebral cortex increases during the first postnatal week (Bandeira et al., 2009). These results confirmed that even during the postnatal period, new neurons are generated.

Another intriguingly result was given by Nottebohm and colleagues (Burd and Nottebohm, 1985; Goldman and Nottebohm, 1983; Paton and Nottebohm, 1984) who demonstrated seasonal cellular plasticity in the brain of adult songbirds. Seasonal cycles of incorporation of new cells occur in the VZ, from where newly formed cells migrate along RG to reach the caudal nucleus of the high vocal center, a structure critical for song production and perception (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla et al., 1990; Nottebohm, 1981).

A milestone was settled in 1992 (Reynolds and Weiss, 1992), when neurons and astrocytes were generated *in vitro* from cells isolated from adult striatum. The importance of this work relies on the fact that it provided the first evidence that cells in the adult central nervous system (CNS) are endowed with differentiative potential not only in the neuronal direction, but also to astrocytic phenotype. Moreover, it provided new evidences regarding the mouse brain plasticity, and concerning the intrinsic ability of adult brain cells to generate new neurons.

Almost at the same time, Lois and Alvarez-Buylla (Lois and Alvarez-Buylla, 1993) demonstrated that explant cultures of proliferative SVZ cells are able to generate both neurons and glia, identifying in the SVZ a new neuronal precursors niche in adult mammalian brain. In the same year, a work from Luskin defined that the specialized region from which an immense number of neurons are generated is the anterior part of the SVZ and that this region appears to constitute a specialized source of neuronal progenitor cells (Luskin, 1993).

Following the identification of a niche of stem cells in the adult CNS, Gage and colleagues were able to isolate and culture cells capable of proliferation and neuronal differentiation from the adult rat hippocampus. Moreover, when grafted into the adult rat brain, these cells integrate in the DG of the hippocampus, retaining the capacity to generate mature neurons (Gage et al., 1995b, 1998).

Studies were then performed to investigate whether the hippocampus, neurogenic niche in rodents and monkeys (Gould et al., 1999), maintain the same features also in humans. Obsrrving post-mortem human brains from cancer patients treated with BrdU for therapeutic purposes, and thus labelling DNA during S phase, and performing immunfluorescent labelling for neuronal markers, it has been demonstrated that there are new proliferating neurons in adult human brain, and these are generated from dividing progenitors in the DG of the hippocampus. This was one of the first breakthrough demonstrating that human hippocampus retain the ability to generate new neurons throughout life (Eriksson et al., 1998). Moreover, cells extracted and cultured from lateral ventricle and hippocampal human biopsies, confirmed that these cells are self-renewing and capable of generating neurons, astrocytes, and oligodendrocytes *in vitro* (Johansson et al., 1999).

The identification of adult NSCs opened new questions about the identity of these cells. Doetsch and colleagues, in 1999, were able to identify in the astrocytic population of the SVZ the NSCs present in the adult CNS. They described that SVZ is composed of four cell types: migrating neuroblasts, immature precursors, astrocytes, and ependymal cells (for a detailed description of these population, please refer to the following section about adult NSC niche). SVZ astrocytes, and not ependymal cells, remain labelled with proliferation markers after long survivals in adult mice and, after elimination of immature precursors and neuroblasts by an antimitotic treatment, SVZ astrocytes divide to generate immature precursors and neuroblasts, to restore the neurogenic population (Doetsch et al., 1999).

A tight connection between neural stem niche and vasculature was demonstrated in 2000, in a work from Palmer. They found that dividing cells within the hippocampal niche are usually disposed in dense clusters associated with the vasculature and roughly 37% of all dividing cells are immunoreactive for endothelial markers. As most of the newborn endothelial cells disappear over several weeks, this suggests that neurogenesis is associated with a process of active vascular recruitment and subsequent remodeling (Palmer et al., 2000).

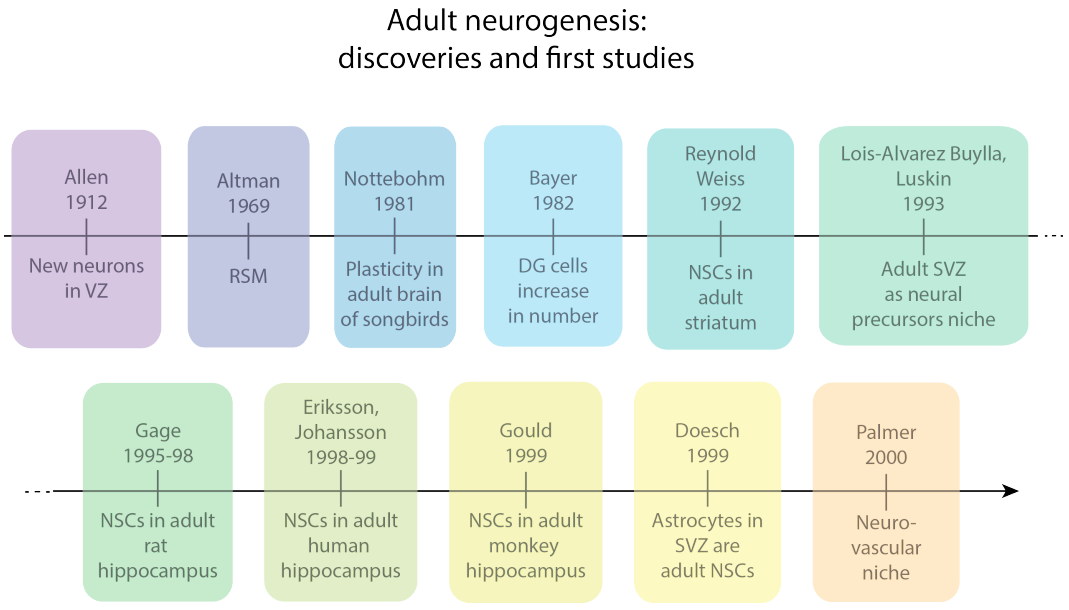

Figure 1.9. Schematic timeline of studies on adult neurogenesis.

This scheme depicts the timeline of some of the important studies and discovery about the adult neurogenesis, starting from the observations of Allen in 1912, to the work of Palmer in 2000.

These brief introductory chapters described the milestones in the discovery of embryonic and adult neurogenesis. In the following chapters, I will analyze more in detailed the properties and the features of the neurogenic niches.

1.1.2 - Adult neural stem cell niches

As already mentioned, neurogenesis in adult mammals, rodents and non-human primates occurs primarily in two regions of the brain: the SVZ and the SGZ of the hippocampus (Figure 1.10). These two classical neural stem cell niches contribute to different populations in the adult brain.

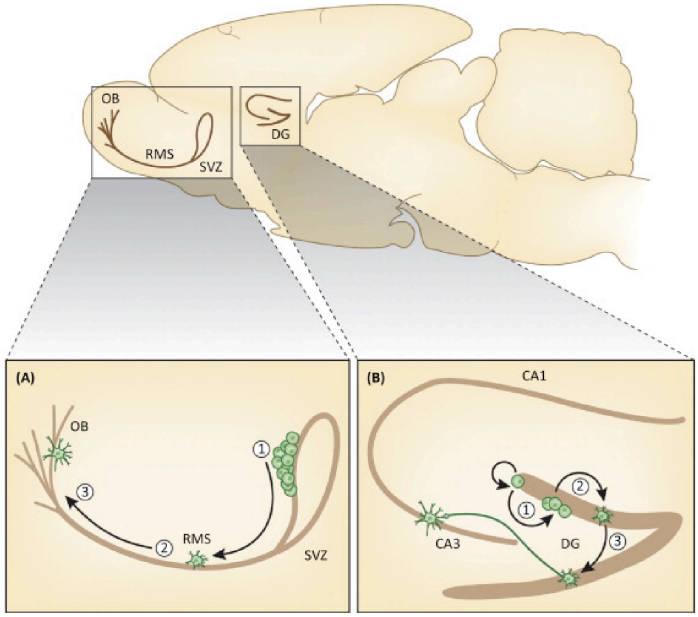

Figure 1.10. Localization and representation of neural stem cell niches in the adult rodent brain.

Inset in (A) represents the SVZ niche, with newborn neurons migrating along the RSM to the OBs; inset in (B) shows the DG niche and the formation of CA3 neurons in the hippocampus. Modified from Borsini et al., 2015.

SUBVENTRICULAR ZONE (SVZ)

Newborn neurons originate from the anterior part of the SVZ, then migrate along the RMS towards the OBs, where they differentiate into granular and perigromerular neurons (Alvarez-Buylla et al., 2002; Encinas and Enikolopov, 2008), *i.e.* interneurons essential for the maintenance and replacement of OB cells.

Multiple cell types coexist in the SVZ, as first described by Fiona Doetsch (Doetsch et al., 1997; Figure 1.11). The majority of cells in the adult SVZ are migrating neuroblasts (type A cells) that continue to proliferate. These cells form an extensive network of tangentially oriented pathways throughout the lateral wall of the lateral ventricle. Type A cells move long distances through this network at high speed

by means of chain migration. The chains of type A cells are ensheathed by slowly proliferating astrocytes (type B cells), the second most common cell type in this germinal layer. The most actively proliferating cells in the SVZ, type C, form small clusters dispersed throughout the network. These foci of proliferating type C cells are in close proximity to chains of type A cells (Doetsch et al., 1997, 1999; García-Verdugo et al., 1998).

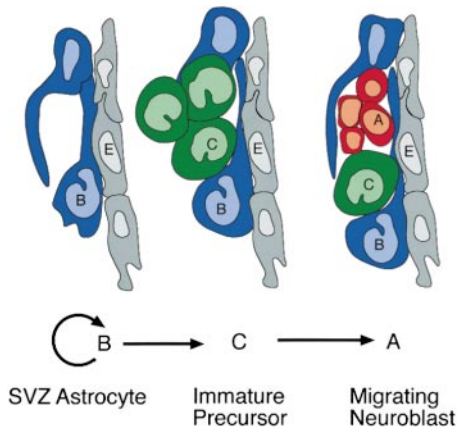

Figure 1.11. SVZ cyto-architecture.

SVZ astrocytes (type B cells, blue) are the primary neuronal precursors in the SVZ. These cells divide to give rise to clusters of type C cells (green), which in turn generate migrating neuroblasts (type A cells, red). Ependymal cells (gray) line the wall of the ventricles and in our experiments did not divide. Modified from Doetsch et al., 1999.

DENTATE GYRUS (DG)

The SGZ of the hippocampus generate newborn neurons that migrate locally in the DG. It is estimated that about 9000 new neurons are generated every day in the rodent DG, corresponding to 0.1% per day of the all granule cell population (Taupin, 2006). There, they settle in the granule cell layer and differentiate into granular neurons, crucial for new learning and memory.

The SGZ, similarly to the SVZ, is composed by different cell types (Figure 1.12). Astrocytes (or type B cells) populate the SGZ and can be distinguished in two sub-populations: radial and horizontal astrocytes (Seri et al., 2004). B cells proliferate to generate the intermediate precursors, or D cells (Seri et al., 2001), which progressively generate more differentiated progeny (D1, D2 and D3). Finally, they mature in granular cell neurons (G cells). Young neurons are located at the SGZ–

granular cell layer (GCL) border and, as they become older, they are displaced deeper into the GCL (Riquelme et al., 2008; Seri et al., 2004).

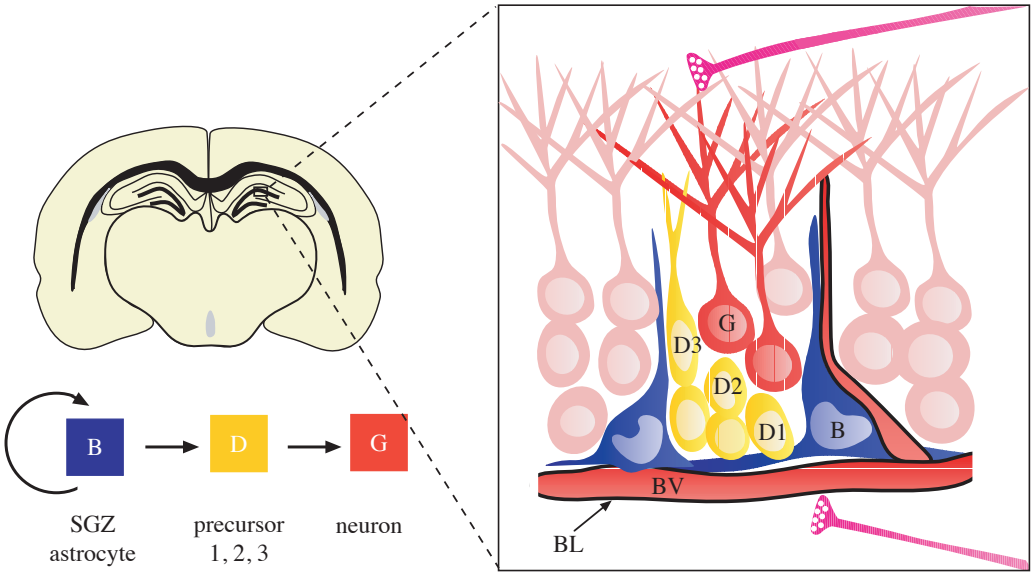

Figure 1.12. Cell types in the adult SGZ.

SGZ astrocytes (B, blue) divide to generate intermediate precursors (type D cells, yellow), which progressively generate more differentiated progeny (type D1, D2, D3), which mature into granule neurons (G, red). In the scheme is highlighted the proximity to the blood vessels. From Riquelme et al., 2008.

A majority of the D cells in the SGZ proliferate in neuroangiogenic foci (Palmer et al., 2000), where neuronal, glial, and endothelial precursors divide in tight clusters. It seems likely that neurogenic hot spots grow and shrink in response to discrete local signals that, given the vascular involvement in neurogenesis, are probably originated from either somatic tissues or the CNS to modulate the production of new neurons (Palmer et al., 2000).

Adult neurogenesis differs from embryonic neurogenesis in several aspects. First, adult neurogenesis proceeds in an environment that is not developing, thus not programmed to promote neurogenesis anymore. Therefore, there is a crescent needs for protection against anti-neurogenic influences of the surrounding tissue and requires the maintenance of a permissive microenvironment (Kempermann et al., 2004). Second, embryonic development shows a precise, massively parallel progression of developmental stages while, in the adult hippocampus, neurons of all

developmental stages can be found, at any given time point (Kempermann et al., 2004).

In conclusion, adult neurogenic niches can be conceptualized as remnants of embryonic signaling centers: they are the source of instructive signals that determine the fate of neighboring stem cells. However, in contrast with stem cells in the developing brain that must cope with a continuously changing environment, adult stem cells are surrounded by a relatively stable niche. Genetic analysis of adult neurogenesis suggests that it is an unstable process, since removal of individual regulatory genes often results in dramatic changes in the behavior of adult stem cells (Urbán and Guillemot, 2014).

1.1.3 - Ectopic neural stem cell niches

New evidences support the hypothesis that not only in response to injury, but also in physiological conditions, ectopic neural stem cell niche are activated in the adult brain (Figure 1.13).

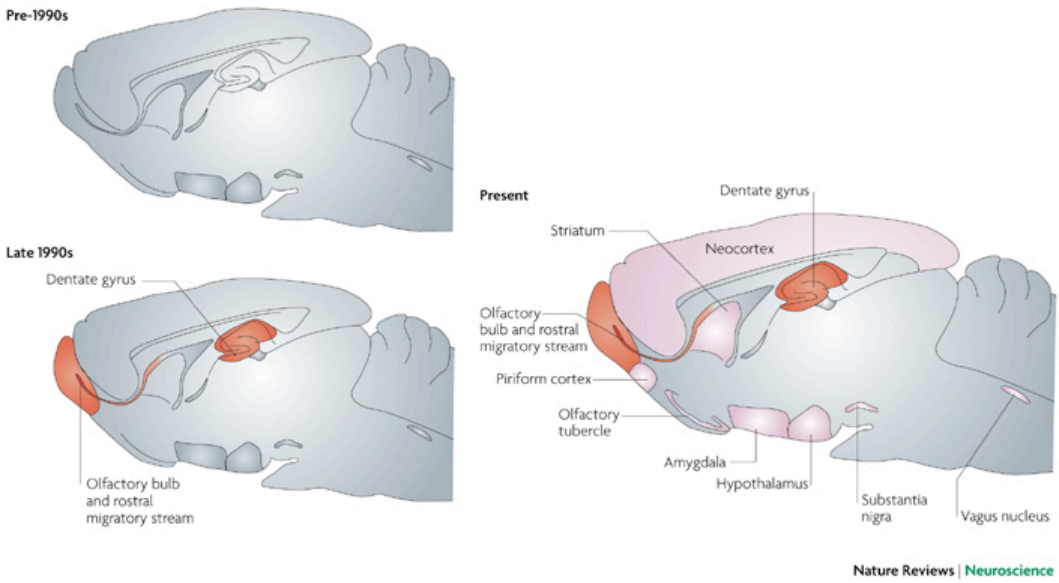

Figure 1.13. Changes in the view of adult neurogenesis in the mammalian brain over the past 15 years.

In the pre-1990s, all regions were categorized as "non-neurogenic" (grey). In the late 1990s, only the DG, SVZ and OBs were categorized as "neurogenic" (red). Today: in pink are indicated the areas where adult neurogenesis is still controversial and on debate, as neocortex, striatum, piriform cortex, amygdala and hypothalamus. From Gould, 2007.

HYPOTHALAMUS AND CIRCUMVENTRICULAR ORGANS

Recent evidences indicate that the hypothalamus hosts a NSC niche at the level of the region lining the third ventricle. Indeed, several reports in different species described the existence of constitutive neurogenesis in the adult hypothalamus (Migaud et al., 2010). It has been demonstrated that multiple factors can influences the proliferation of new cells in the hypothalamus, e.g. photoperiod (in hamster, Huang et al., 1998), estrogens and social interactions (in voles, Fowler et al., 2002, 2005).

Moreover, a detailed description of the circumventricular organs of rat and mouse as novel NSC niche has been provided, based on the expression of markers such as nestin, GFAP and Sox2 in cells expressing also the proliferative marker Ki67 and that incorporated BrdU (Bennett et al., 2009).

STRIATUM

Neurogenesis in the adult striatum has been described in different species, as rat, rabbit and macaque. Indeed, Dayer observed the generation of GABAergic interneurons in the adult rat striatum (Dayer et al., 2005), while in adult rabbit the generation of new neurons has been found mainly in the caudate nucleus (Luzzati et al., 2006). In this work, they stated that this phenomenon is independent from that occurring in the adjacent SVZ and mostly attributable to the activity of clusters of proliferating cells located within the parenchyma of this nucleus. Moreover, studies in adult new world monkeys demonstrated that proliferative cells were encountered within both the dorsal and the ventral striatum (Bédard et al., 2002a, 2006).

CEREBELLUM

Studies in peripuberal rabbits demonstrated that cerebellum shows remarkable neurogenesis, and that this phenomenon persist to a lesser extend until adulthood. The subpial layer indeed hosts proliferating elements that form a single, non-continuous layer independent from the meninges, and these cells persists and incorporated BrdU 2 weeks and, to a lesser extend, also 2 months after birth. The production of these new progenitors, including neuronal precursors and glial-like cells, continues at high rates up to and beyond puberty (Ponti et al., 2006, 2008).

OLFACTORY BULBS (OBs) AND MUCOSA

New neuroblasts generated in the SVZ migrated towards the RSM and settle in the OBs. However, a new group of cells has been identified as source of neuronal and not-neuronal cells in the olfactory epithelium, *i.e.* the horizontal basal cells. Indeed, they are shown to be competent to regenerate and restore both neuronal and not-neuronal lineages in the olfactory neuroepithelium, and they serve as reservoir of long-lived progenitors that remains largely quiescent during normal neuronal turnover (Leung et al., 2007). Moreover, other studies demonstrated that the globose

basal cells are also able to restore olfactory epithelium after chemical ablation, as they are multipotent, transit amplifying cells and immediate neuronal precursors (Mahalik, 1996; Newman et al., 2000). It has been shown that, in new world monkeys, a significant number of $BrdU^+$ cells deviate from the main stream of the RMS. Instead of reaching the olfactory bulb, these cells migrate ventrally into the olfactory tubercle, where they express a mature neuronal phenotype (Bédard et al., 2002b).

Confirmation of the stemness and the regenerative potential of this accessible niche is given by a study of 2005, where they demonstrate that extracted olfactory ensheathing cells from nasal biopsies can be transplanted to injured spinal cord (SC), promoting regeneration and remyelination of descending pathways through the site of injury, and the return of motor function (Murrell et al., 2005).

RETINA

In mammals, the two functional components of the retina, the inner neural retina and the outer retinal pigmented epithelium, achieve their development by early postnatal period, and no additional retinal cells are produced thereafter. However, independent works showed that single pigmented cells from the ciliary epithelium of mouse retina could clonally proliferate *in vitro* to form sphere colonies (Ahmad et al., 2000; Tropepe et al., 2000), and these colonies have self-renewal ability and mulitpotency. Indeed, they express gene found in rod photoreceptors, in bipolar neurons and Müller glia. Few years later, retinal stem cells, mitotically quiescent *in vivo*, have been identified in the ciliary bodies of other mammalian species, including human (Coles et al., 2004; Gu et al., 2007; MacNeil et al., 2007; Mayer et al., 2005).

In addiction, retinal stem cells have been identified also in the iris (Haruta et al., 2001), in the sclera (Arsenijevic et al., 2003), and Müller glial cells, thus representing a potential source of stem cells (Poitry et al., 2000; Simón et al., 2012).

SPINAL CORD (SC)

Two different models have been proposed regarding the localization of NSCs in the adult SC. In the first, a slowly proliferating stem cells resides in the ependymal layer of the central canal (Beech et al., 2004; Horner et al., 2000); in the second, stem cells and glial progenitors are suggested to exist in the parenchyma of the SC (Obermair et al., 2008), and to be independent of the proliferative ependymal.

Accordingly to the first model, stem cells in the ependymal layer divide asymmetrically and the daughter cell migrates to the outer circumference of the SC, and begins to divide more rapidly. In this way, there is a net division between slowly proliferating cells in the central canal and faster dividing cells in the outer SC. Cells from the ependymal zone of the central canal can be cultured *in vitro* as neurospheres (Hamilton et al., 2009). These cells, in SC sections, express vimentin, and Ki67 proliferative cells are primarly found in this zone and are distributed in a dorsal to ventral gradient, often found in doublets and close to the blood vessels.

The second model describes the presence of progenitors in the SC parenchyma. Indeed, neuroblasts have been identified in the adult SC parenchyma and here the progenitor cells give rise to immature neurons expressing DCX, GAD65/67 and GABA (Shechter et al., 2007).

Moreover, cell transplantation studies have demonstrated that, although NSCs from the SC will differentiate into glia when re-implanted in the region of origin, they are able to differentiate into neurons when grafted in the hippocampus (Shihabuddin et al., 2000).

CEREBRAL CORTEX

Although it is believed that no new neurons are generated from cortical cells, there are some evidences indicating that also the cortical tissue may retain some neurogenic properties in the postnatal period.

One of the examples is given by evidences that show how SVZ-derived cells in the rat retain the capacity for division after migrating from their initial site of generation. These cells, expressing a neuron-specific tubulin recognized by the antibody Tuj1, are suggested to have made a commitment to become neurons before becoming postmitotic (Menezes et al., 1995).

Moreover, a new source of neuronal and glial progenitors has been found in the marginal zone of the developing cerebral cortex, and this niche presents properties notably distinct from those of VZ and SVZ progenitors in terms of molecular characteristics, size and identity of their clonal progeny (Costa et al., 2007).

Despite these intriguing results, data produced by analysis of integration in DNA of ^{14}C generated by nuclear bomb tests during the Cold War, in DNA to establish the age of neurons in the major areas of the human cerebral neocortex, it has been

shown that under normal conditions non-neuronal cells turn over while neurons in the human cerebral neocortex are generated perinatally and not at detectable levels in adulthood (Bhardwaj et al., 2006).

However, the recognition of the cerebral cortex as neurogenic tissue is still debated and the issue needs to be investigated.

1.1.4 - Novel neurogenic mechanisms: direct conversion and reprogramming

Besides the discovery of ectopic adult NSC niches, also novel mechanisms supporting adult neurogenesis outside the "classical" niches have been discovered. These are based on recent findings about the presence of quiescent NPs in the adult brain and the mechanism of direct conversion of NPs into neurons, without a prior proliferative step.

POOLS OF QUIESCENT NSCs

Recent studies reported that a subset of ventricular RGCs, generated between E13.5 and E15.5, becomes quiescent and act as neural stem cells in the adult brain (Fuentealba et al., 2015; Gage and Temple, 2013); these cells provide a direct link between progenitors in the embryo and postnatal/ adult NSCs that are retained in the walls of the lateral ventricles and continue producing neurons for the OBs (Fuentealba et al., 2015).

Moreover, it has been described that stem cells in the neuro-vascular compartment, in particular when direct cell-cell contact between NSCs and endothelial cells is allowed, maintain stemness features of NSCs, preventing them to differentiate. Indeed, endothelial ephrinB2 and Jagged1 proteins enforce quiescence and promote stem cell identity, identifying in the vasculature a critical niche compartment for stem cell maintenance (Ottone et al., 2014).

In the mouse hippocampal niche, two different population of quiescent progenitors have been described; one directly activated by high-KCl induced depolarization and the other activated by norepinephrine (Jhaveri et al., 2015). Thus, adult hippocampus contains phenotypically similar but stimulus-specific populations of quiescent precursors, which may give rise to neural progeny with different functional capacity. Moreover, a quiescent population of NSCs have been described also in the guinea pig striatum, and these cells become active only following the weaning (Luzzati et al., 2014).

Thus, an increasing number of studies are demonstrating that, in the adult brain, co-exist active and quiescent NSCs. These cells can activate in different phase of life and following different stimuli. The presence of quiescent progenitors in the adult mammalian brain may represent a novel source of stem cells available after traumatic events.

DIRECT CONVERSION AND REPROGRAMMING

New studies are reporting the possibility to reprogram and direct convert the fate of non-neuronal cells into neuronal phenotype, without passing through the symmetric, neurogenic division step required for neuronal production.

An example is given by the possibility to reprogram astrocytes into neurons in mice. In fact, it has been shown that, upon Notch1 signaling regulation, striatal astrocytes may reveal a latent neurogenic potential (Magnusson et al., 2014).

Another study reports the conversion of mouse embryonic and postnatal fibroblasts into functional neurons *in vitro*. These induced neuronal cells express multiple neuron-specific proteins, generate action potentials and form functional synapses upon the administration of Ascl1, Brn2 and Myt1l in culture (Vierbuchen et al., 2010).

Götz and colleagues reported neuronal features in mammalian astroglia overexpressing PAX6 (Heins et al., 2002), demonstrating an important role of Pax6 as intrinsic fate determinant of the neurogenic potential of glial cells. They then showed that retrovirus-mediated expression of the transcription factors Sox2 and Ascl1 can induce the conversion of genetically fate-mapped NG2 glia into induced doublecortin $(DCX)^+$ neurons in the adult mouse cerebral cortex, following stab wound injury *in vivo*. In contrast, lentiviral expression of Sox2 in the unlesioned cortex failed to convert oligodendroglial and astroglial cells into DCX^+ cells (Heinrich et al., 2014).

Moreover, evidences in zebrafish suggested a possible new mechanism of neurogenesis, based on direct differentiation of neural precursors to neurons without the cell division-step. Indeed, new neurons are generated by both direct conversions of stem cells into postmitotic neurons and via intermediate progenitors amplifying the neuronal output following brain injury. These results reveal changes in the behavior of stem cells underlying generation of additional neurons during regeneration (Barbosa et al., 2015).

All these evidences open new possibilities regarding the generation of cortical neurons during the postnatal period. Indeed, it is known that neuronal-committed progenitors expand and proliferate during the embryonic period (see paragraph

1.1.1), and no proliferating cortical neurons in the postnatal period have ever been reported (Altman, 1969). The newly described mechanisms of direct conversion and the presence of quiescent populations in the adult brain may represent a novel mechanism and source of neuronal precursors available following injury and traumatic events, increasing the possibility of recovery due to a therapeutic action of endogenous stem cells.

1.2 - Meninges

Meninges have originally been viewed as specialized membranes wrapping the CNS and protecting it from injuries. However, recently, this view has been modified accordingly to new increasing evidences about the role of meninges in brain development and homeostasis (Siegenthaler and Pleasure, 2011a) and as a niche for stem/ progenitor cells (Bifari et al., 2009, 2015)

1.2.1 - Anatomy and histology

Meninges are composed by three layers (dura, arachnoid and pia mater) which together envelop the CNS and penetrate its parenchyma (McMenamin et al., 2003; Figure 1.14).

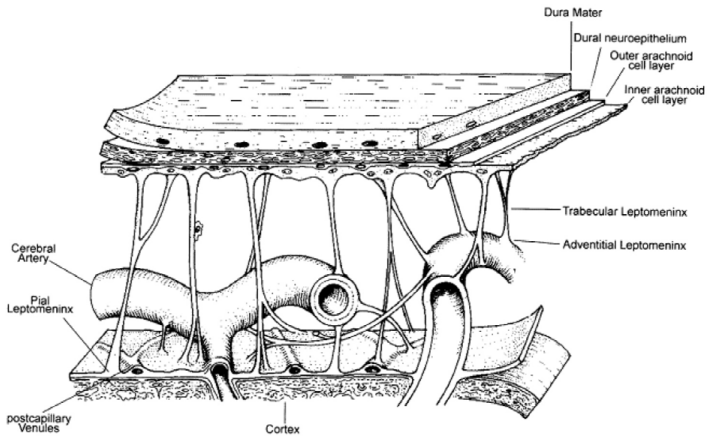

Figure 1.14. Schematic drawing of the meningeal layers.
The dura mater and the arachnoid cell layers lie in tight contact with each other; underneath, arachnoid trabeculae cross the subarachnoid space, where the CSF flows. The thin layer of the pia mater covers the neural parenchyma and penetrates it along blood vessels. Modified from McMenamin et al., 2003.

The dura mater is the outer and thicker layer of the meninges. It is tightly connected to the cranial and vertebral bones and composed by three different layers: the endosteal layer, the meningeal layer and the border cells layer. The endosteal layer lies in tight contact with the skull and the vertebrae of the SC and is composed mainly by fibroblast-like cells and extracellular collagen fibers, which are responsible

for the resistance that characterizes this layer. Beneath the endosteal layer, the meningeal layer confers the dura its flexibility, due to the high presence of fibroblasts and the low content of extracellular matrix components. Finally, the border cells layer lies in direct contact with the arachnoid, composed of a thin layer of flat cells connected by desmosomes and extracellular space filled with amorphous material (Adeeb et al., 2012; Greenberg et al., 1994; Nabeshima et al., 1975; Patel and Kirmi, 2009).

Between the dura mater and the parenchyma of the CNS lie the arachnoid and pia mater, which together are called leptomeninges; these two layers surround blood vessels and nerves as they penetrate the brain and spinal cord parenchyma. The arachnoid is in direct contact with the dura mater through its outer mesothelial layer, which is composed of few layers of flat cells separated by gaps; tight junctions are regularly spaced and are fundamental to the barrier function of this layer. Underneath the outer mesothelial layer, the inner reticular layer is less organized and is composed of cells whose processes are connected by gap junctions and desmosomes and are surrounded by collagen fibers and small cisternae (Nabeshima et al., 1975; Vandenabeele et al., 1996).

A distinguishing feature of the leptomeninges is the subarachnoid space, located between the arachnoid and the pia mater; this space contains the cerebrospinal fluid (CSF) that envelopes the brain and SC and is traversed by arachnoid trabeculae, a peculiar feature that gives its name to this layer. These structures are composed by a collagen core and are covered by leptomeningeal cells: they divide the subarachnoid space into compartments and facilitate the flow of CSF (Alcolado et al., 1988). The CSF is in fact produced in the brain ventricles by the choroid plexi and is subsequently distributed to the CNS thanks to the ciliated ependymal cells of the ventricles, the pressure gradients and the presence of one-way valves (the arachnoid villi) that project in the subarachnoid space (Barshes et al., 2005).

Underneath the subarachnoid space lies the pia mater, that can be divided into a vascular layer, composed by a network of minute blood vessels intermingled with collagenous fibers, and an avascular layer composed of reticular elastic fibers separated from the CNS parenchyma by the basement membrane of the glia limitans, to which RGCs (at embryonic stages) and astrocytic endfeet (in the adult CNS) are attached; blood vessels from the subarachnoid space lie directly on the pia mater

before entering the brain and SC (Millen and Woollam, 1961). Additionally, the pia mater creates a peculiar extracellular space called Virchow-Robin space: it follows the blood vessels as they enter in the CNS parenchyma and reflects over them, therefore separating the perivascular space from the subarachnoid space (Barshes et al., 2005). The thin pial barrier envelops the CNS parenchyma, the subpial space, the neural extracellular spaces and the perivascular space and separates this compartment from the subarachnoid space (Figure 1.15).

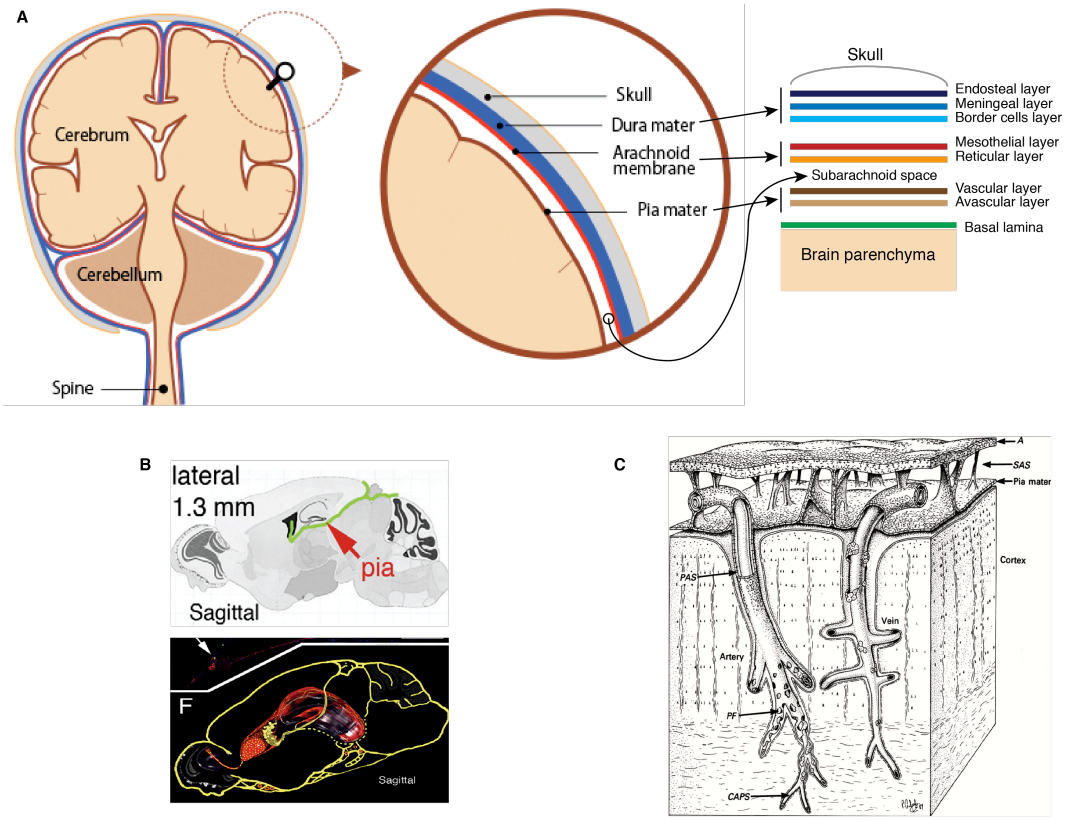

Figure 1.15. Schematic representation of the meningeal layers.

A, This draw depicts the localization of the meninges covering and penetrating the CNS. The pia mater is indicated in brown and it's in close contact with the basal lamina (green) of CNS parenchyma. The pia mater is divided from the arachnoid (red/orange) by the subarachnoid space; the dura mater (blue/light blue) is the outer layer, in direct contact with the skull. Modified from Department of Neurosurgery Tokai University Hospital. **B,** Diagram representing the meninges and meningeal substructure (green, yellow) penetrating inside the brain. Modified from Mercier and Arikawa-Hirasawa, 2012. **C,** Diagram demonstrating the relationship between pia mater and intracerebral blood vessel. Modified from Pollock et al, 1997.

Meninges not only cover the brain, but also enter it by projecting between brain substructures. A major meningeal projection is represented underneath the hippocampal formation, and it is continuous with the choroid plexus stroma (Mercier and Hatton, 2003); others are around and within the SC, circumventricular organs and within the cranial nerves (Mercier and Hatton, 2001). Small meningeal projections are identified in sheaths of blood vessels (adventitia).

1.2.2 - Embryology

The first meningeal layer is identifiable early in development in chicken, at E2 (Etchevers et al., 1999), in mouse between E9 and E10 (McLone and Bondareff, 1975) and in humans during the 4th gestation week (O'Rahilly and Müller, 1986).

Development of pia-arachnoidal membranes in the mouse occurs in four stages: the first (E10-E13) follows closure of the neural tube and is a period of initial vascularization of the developing telencephalon; the second (E14-E16) is a period of delineation during which the limits of the subarachnoid space are defined; the third (E17 to birth) is a period of ensheathment of pia-arachnoidal blood vessels; and the fourth (birth to P21) includes addition of smooth muscle to larger vessels, the appearance of macrophages in the subarachnoid space, and a general increase in extracellular collagenous and elastic fibers (McLone and Bondareff, 1975).

Human meninges originate from a mesenchymal tissue that surrounds the neural tube and was termed "meninx primitiva" in 1898 (Salvi, 1898). The leptomeningeal matrix is initially composed of mesenchymal neural crest cells and a primitive vascular net of endothelial cells. Johnston first observed a potential contribution of neural crest cells to the forebrain meninges, which includes pericytes and connective tissue cells (Couly et al., 1995; Johnston, 1966). Thus, the cephalic neural crest cells penetrates the forebrain neuroepithelium together with vascular buds made up of endothelial cells. This double origin of the leptomeninges from the neural crest and paraxial mesoderm is exclusively found in the forebrain; current knowledge suggests that, in the rest of the CNS, the meninges are entirely of mesodermal origin (Couly et al., 1992; Siegenthaler et al., 2009).

The primitive meningeal tissue subsequently divides into two layers: an inner layer, or endomeninx, that generates the leptomeninges, and an outer layer, also

called ectomeninx, which generates the dura mater. The pial meningeal layer, the inner one, is in thigh contact with the blood vessels and with the pial basement membrane. The other two layers, the arachnoid and the dural layer, are single layers of cells beneath the calvarian mesenchyma (Couly and Le Douarin, 1987; Jiang et al., 2002, Figure 1.16).

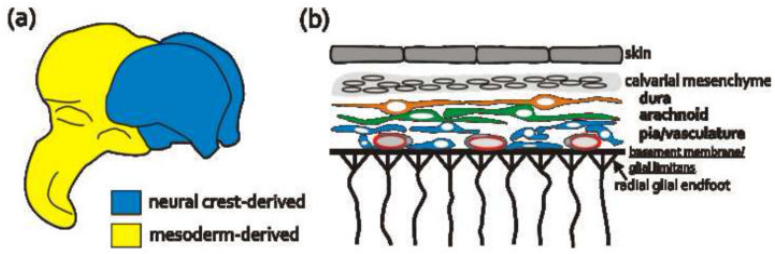

Figure 1.16. Origin and structure of fetal meninges.

Panel in (a) shows that the forebrain meninges are neural-crest derived, whether the meninges covering the other regions of the brain and spinal cord are mesoderm-derived. Panel in (b) shows the different cellular populations in meninges. Modified from Siegenthaler and Pleasure, 2011.

Meninx primitiva also participates in the formation of the extracerebral vasculature: the extracerebral or meningeal vascular compartment is present already at the 7th week of gestation in humans and includes venous sinuses of the dura, arachnoid veins and arteries and pial capillary plexus. From the pial capillary plexus, which covers the entire surface of the CNS parenchyma, originate the blood vessels that perforate the glia limitans and reach the paraventricular zone of the brain. Through the Virchow-Robin space of these perforating blood vessels, the CNS parenchyma communicates with the cells and fluids present in the meninges (Marín-Padilla and Knopman, 2011).

1.2.3 - Role of the meninges in CNS development

Meninges are also known to be involved in the regulation of CNS development and homeostasis. Indeed, they play a pivotal role in the formation of the cerebral vasculature during embryonic development and are required for neuroepithelium viability. As already mentioned, in the developing forebrain mesodermal cells give rise to endothelial cells that form the blood vessels that penetrate in the neuroepithelium, while neural crest cells contribute to leptomeninges and forebrain

formation, providing pericytes and connective tissue cells. Experimental ablation of the neural crest that give rise to the telencephalic and diencephalic meninges impedes proper meningeal development; this, in turn, causes extensive apoptosis of the neuroepithelium of the forebrain (Etchevers et al., 1999). In the same study, Etchevers and colleagues showed that paraprosencephalic mesoderm is able to replace neural crest-derived pericytes, leading to correct formation of leptomeniges; rescue of leptomeningeal development allows proper formation of the neuroepithelium.

INTERACTION WITH RADIAL GLIAL CELLS (RGCs)

The interaction between meningeal cells and RGCs in the developing brain is fundamental for proper organization of the cortical neuronal layers. Disruption of meningeal integrity during late embryonic development provokes abnormal localization of RGCs (Sievers et al., 1994) and altered lamination in the cerebellar cortex of newborn mice (von Knebel Doeberitz et al., 1986); similarly, ablation meningeal cells provokes abnormal morphogenesis in the DG of the hippocampus (Hartmann et al., 1992). Alterations of brain development after destruction of meningeal cells are primarly due to disorganization of the pial basal membrane. RGCs extend their process through the developing cortex to guide migration of neuroblasts; damage to the pial basal lamina prevents RG attachment and results in cortical plate dysplasia and formation of ectopic aggregations of neurons beyond the pial membrane (Halfter et al., 2002). In addition, interaction between pial and RGCs is necessary for proliferation and amplification of the RG in the early phases of cortical development (Radakovits et al., 2009).

SDF-1 AND THE HOMING PROCESSES

NSCs are shown to migrate to their niche, through a process called homing, guided by specific signalling. Stromal-derived factor 1 (SDF-1, also known as CXCL-12) and its receptor CXCR4 are involved in the homing process of different populations of stem cells and in forebrain development. SDF-1 directs hippocampal dentate granule cells (Bagri et al., 2002), Cajal Retzius cells (Paredes et al., 2006), cerebellar granular neurons (Ma et al., 1998; Zou et al., 1998), and cortical interneurons (Stumm et al., 2003; Tiveron et al., 2006) to their correct locations

within the brain (Kokovay et al., 2010). Moreover, neuroblasts in the adult SVZ migrate out of the germinal zone toward sites of ischemic injury after stroke in response to SDF-1 release (Arvidsson et al., 2002; Hill et al., 2004; Yamashita et al., 2006), becoming associated with the vasculature (Ohab et al., 2006; Robin et al., 2006; Thored et al., 2006). Upon transplantation of adult SVZ progenitors, they integrate into the host SVZ and migrate toward blood vessels thanks to the SDF-1-CXCR4 axis active in SVZ, vascular plexus and ependymal cells (Kokovay et al., 2010). Moreover, SDF-1 does not notably stimulate movement of SVZ-type B cells, which include the more primitive, quiescent stem cells; in contrast, SDF-1 stimulates chemotaxis of activated SVZ-type C cells and their adhesion to endothelial cells. Furthermore, SDF-1 promotes motility of type A neuroblasts, consistent with endothelial-derived SDF-1 stimulating their migration to the olfactory bulb (Kokovay et al., 2010a).

Meninges produce and secrete SDF-1, which guides Cajal-Retzius cells migration to the subpial MZ; these cells, in turn, secrete the glycoprotein reelin that regulates lamination of neuronal cells in the cortex (Borrell and Marín, 2006). At later stages of development, SDF-1 from the meninges attracts inhibitory interneurons from their site of origin in the ventral forebrain to the MZ migratory stream in the dorsal forebrain (López-Bendito et al., 2008). Meningeal SDF-1 is also involved in the regulation of neural progenitor cells localization: granule cell progenitors of the developing cerebellum proliferate in the external granule cell layer before migrating inwards; SDF-1 secreted by the meninges regulates their position and contributes to stimulate proliferation, in cooperation with Shh (Klein et al., 2001).

Our group recently demonstrated that meningeal-derived neurospheres from adult spinal cord express SFD-1 and its receptor CXCR4, and the expression of SDF-1, but not of CXCR4, decrease after injury (Decimo et al., 2011). Moreover, the SDF-1-CXCR4 axis was identified *in vivo* in rat brain meninges at different stages of development, from embryonic period up to adulthood (Bifari et al., 2015).

SECRETION OF REGULATORY MOLECULES

It has been shown that meninges overlying the primitive forebrain secrete retinoic acid (RA), which is a regulatory molecule necessary for early cortical neurons generation. Indeed, dorsal forebrain meninges communicate with the adjacent RG

endfeet and influence cortical development through the activity of RA (Siegenthaler et al., 2009). Moreover, meninges covering the dorsal hindbrain participate in anterior hindbrain development by generating a dorsal-ventral gradient of meningeal-derived RA (Zhang et al., 2003).

Meninges have been shown to be involved also in regulation of corpus callosum development. Meninges in fact secrete BMP7, an inhibitor of callosal axon outgrowth. This activity is overcome by the induction of expression of Wnt3 by the callosal pathfinding neurons, which antagonize the inhibitory effects of BMP7. Alterations in the participation of the meninges to this process cause callosal agenesis in the case of hyperplasticity of meningeal tissue, or increased callosal thickness in the case of defects in the meninges (Choe et al., 2012).

Thus, meninges secrete molecules that create permissive and nonpermissive environments to migrating neurons, regulating the correct development of the CNS (Lu et al., 2002).

PRODUCTION OF EXTRACELLULAR MATRIX (ECM) COMPONENTS

The role of meninges in CNS development also involves production and interaction with different components of the extracellular matrix (ECM). Meningeal cells have in fact been shown to produce a high variety of ECM components, such as collagen type I, III and IV, fibronectin, laminin, tenascin, heparan sulfates and nidogen (Montagnani et al., 2000; Sievers et al., 1994). The importance of the production of basal lamina constituents by meningeal fibroblasts has been clearly evidenced; indeed, deletion of focal adhesion kinase in these cells disrupts pial membrane integrity and provokes excessive neuronal migration to the MZ (Beggs et al., 2003).

Moreover, a recent study by our group evidences the presence of laminin and N-sulfated heparan sulfates in vascular basement membranes and in fractones, in rat brain meninges at different developmental stages, from embryonic up to adulthood (Bifari et al., 2015). Similar structures have been shown to play a significant role in binding and activating heparin-binding factors, such as bFGF, in the neurogenic niche of the SVZ, therefore participating in the regulation of cell proliferation in the adult brain (Douet et al., 2013).

1.2.4 - Meninges and CNS homeostasis

Meninges cover and penetrate the brain at every level of its organization. They project as membranes between the major brain structures (*i.e.* hemispheres, hippocampus and thalamus), as sheaths of blood vessels, as stroma of the choroid plexus and also as the non-neural roof of the ventricles (Mercier, 2003). A major meningeal substructure extends underneath the hippocampus and is continuous with the choroid plexus stroma (Mercier and Arikawa-Hirasawa, 2012). This implies that the different cell populations residing in the meninges are able to contact the cells that compose the CNS parenchyma. Astrocytes have emerged as the major component of a potential network of communication and signalling between CNS neurons and extraparenchymal cells: astrocytes are found intermingled with neuronal cells in both grey and white matter, where they separate and support neurons and form a loose network with their processes; a subpopulation of astrocytes (radial astrocytes) can be found at the MZ of the CNS parenchyma, where they form a layer called glia limitans. Interestingly, both astrocytes of the glia limitans and meningeal cells express high density of gap junctions, such as Cx26, Cx30 and Cx43 (Mercier and Hatton, 2001; Spray et al., 1991), suggesting that an extensive communication exists between both intra- and inter-populations. Meningeal cells and astrocytes have been shown to produce and secrete growth factors and cytokines, which act on nearby cells (Althaus and Richter-Landsberg, 2000; Franzen et al., 1999). In addition, both astrocytes and meningeal cells face the pia mater basal lamina, which is composed by different ECM components, such as collagen, laminins, heparan-sulfated proteoglycans, fibronectin and chondroitin sulfate proteoglycans, that play a fundamental role in signal transduction of growth factors and cytokines during brain development; recent studies showed that participation in regulation of neural stem cells niches such as SVZ through the binding and activating action of fractones is maintained in adult life (Douet et al., 2013).

1.3 - Meninges as potential NSC niche

Over the last years, new and unexpected roles for meninges have emerged (Bjornsson et al., 2015; Decimo et al., 2012a; Richtsmeier and Flaherty, 2013), from just a mere protective layer surrounding the CNS to a new potential niche hosting NSCs.

1.3.1 - Meningeal tissue and the niche environment

Meninges form a complex microenvironment endowed with soluble trophic factors, extracellular matrices and cells playing fundamental roles in both skull and brain development (Bjornsson et al., 2015; Richtsmeier and Flaherty, 2013).

Supporting the concept of meninges as putative neural stem cell niche, are the peculiar morpho-functional properties of meninges themselves. Indeed, meninges may have the potential to modulate stem cell homeostasis since they contain laminin-enriched ECM organized in fractones (Mercier et al., 2002), N-sulfated heparan sulphate molecules (N-sulfated HS), functional gap junctions and secrete several trophic factors (Bifari et al., 2015; Mercier et al., 2002). More recently, evidences have been presented for the presence of telocytes in meninges and choroid plexus. Telocytes are interstitial cells that appear to be in close contact with stem cells and to be able to regulate the stem cell niche by generation of intercellular signals (Popescu et al., 2012).

These observations, together with the consideration about the fundamental role of meningeal cells during brain development, the presence of cells expressing markers of stemness and their activation following CNS injury (please refer to next paragraph for detailed explanation), opened new questions about whether meninges possess the features described for canonical NSC niches (Bjornsson et al., 2015) and whether these features also persist at the end of the developmental period.

Thus, our group analysed by morphological, molecular and biochemical criteria: i) the number and the proliferation rate of leptomeningeal cells under different developmental conditions; ii) the presence and the distribution of cells expressing neural progenitor markers; and iii) the distribution of some of the known extracellular components of neural niches (Bifari et al., 2015).

We first assessed the proliferation rate of meningeal cells during development. We observed that the expression of the proliferation marker Ki67 was present in all

the analysed stages, during development (E20) and in the postnatal phase (P0, P15), until adulthood (6-8 weeks and up to 24 weeks), meaning that meninges are a high proliferative tissue up to the adult stage. Then, we showed that rat leptomeninges harbor a population of cells expressing the undifferentiated neural precursor markers nestin, vimentin and Sox2 during development up to adulthood. At all time-points, a small fraction of meningeal cells also expressed DCX, as for neuronal precursor cells and immature neurons in embryonic and adult cortical structures. NSC niches are characterized by the presence of ECM components and chemotactic factors (Kerever et al., 2007; Kokovay et al., 2010a); thus, we assessed the presence of laminin and N-sulfated HS, a member of he glycosaminoglycan family know to bind and concentrate growth factors as FGF2 and EGF (Mercier and Arikawa-Hirasawa, 2012; Yayon et al., 1991). We identified the presence of N-sulfated HS in laminin-lined interface with parenchyma and in fractones (Mercier et al., 2002), specialized ECM structures that appeared like aligned immunoreactive puncta along the meninges. Fractones of the parietal meninges were similar to fractones described in SVZ (Mercier et al., 2002). In line with these findings, we detected gene expression of the growth factor receptors FGFR1 and EGFR at all time point of analysis, as well as the presence of the chemotactic factor SDF-1 and its receptor CXCR4 (Bifari et al., 2015).

Collectively, these data indicates that similar to the SVZ, leptomeninges host a subset of cells expressing markers of undifferentiated, proliferating and differentiating neural precursors and persisting in adulthood. Moreover, the identification of receptors for trophic factors, of ECM components and chemotactic factors known to be involved in homing, movement, proliferation and differentiation of progenitor cells strengthens the idea that the niche function of meninges is not limited to conditions associated to diseases, such as injury or ischemia (Decimo et al., 2011; Nakagomi et al., 2012), but also present in normal physiological conditions (Bifari et al., 2015).

1.3.2 - Neurogenic potential of the meningeal cells *in vitro*

In support to the described data about meningeal tissue as a suitable microenvironment to host NSCs, several works reported the neurogenic potential of meningeal cells *in vitro*.

Our group was the first that suggested to name meningeal cells "leptomeningeal stem/ progenitor cells" (LeSCs) because of the persistence of these cells up to adulthood, their proliferation capability *in vitro* and their differentiation potential into neuronal cells *in vitro* and *in vivo* (Bifari et al., 2009).

Indeed, cells extracted from young adult rat brain and SC meninges could be cultured as neurospheres, and expanded *in vitro* up to several months (Bifari et al., 2009; Decimo et al., 2011; Figure 17). Similarly to SVZ-derived neurospheres (Bez et al., 2003), LeSC- derived neurospheres consisted of different cell types, including nestin-positive cells, neuronal $MAP2^+$ cells and glial $GFAP^+$ (Bifari et al., 2009; Decimo et al., 2011, Figure 1.17). Moreover, expanded cells retained nestin positivity as a homogeneous population and expressed CD90, a non-specific marker for neural and stromal cells (Gupta et al., 2006)

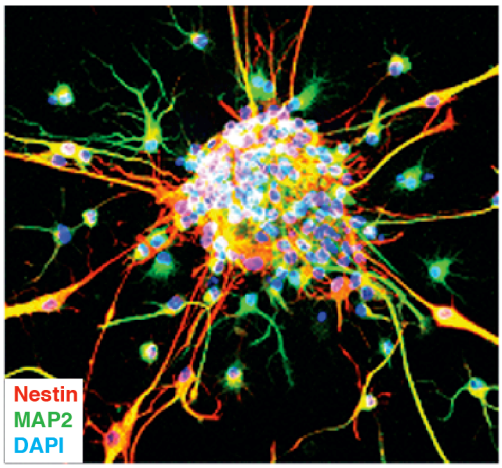

Figure 1.17. LeSC- derived neurosphere.

Confocal image of a LeSC-derived neurosphere, stained with MAP2 (green), nestin (red) and DAPI (blue) (B). Modified from (Bifari et al., 2009).

When cultured in specific differentiating medium, LeSC showed an increase of MAP2 levels in parallel to a decrease of nestin expression, demonstrating how LeSC can be cultured, expanded and differentiated into neurons at high efficiency. $MAP2^+$ cells were derived from replicating cells are shown by experimetns using BrdU and immunocytochemistry. Moreover, LeSC-derived neurons have been shown to be excitable and to express functional voltage-depedent calcium channels (Bifari et al., 2009; Decimo et al., 2011). SC-LeSCs displayed also a differentiation potential to mature oligodendrocytes, as they progressively expressed pre- (NG2, A2B5, and

PDGFRα, early- (O4), or myelinating (GalC and myelin basic protein, MBP) oligodendrocyte markers and acquired oligodendrocyte morphology (Decimo et al., 2011).

In order to confirm the differentiation potential of meningeal cells also *in vivo*, brain-LeSCs were expanded and transplanted in adult rat hippocampus. Cells were present in the pyramidal layer, stratum oriens and DG of the hippocampus 4-8 weeks after transplantation, and most of them expressed MAP2 a week after the injection.

These results gave the first confirmation about the neurogenic potential of LeSC-derived cells both *in vitro* and, surprisingly, after transplantation *in vivo* (Bifari et al., 2009; Decimo et al., 2011).

A interesting work was published showing the presence of nestin$^+$ cells also in human meninges during fetal development, appearing for the first time at the 7th week of gestation (Petricevic et al., 2011).

1.3.3 - Meningeal response and activation following injury and in neurodegenerative diseases

Our group identified a population of stem/ precursors cells in SC meninges, endowed with self-renewal and proliferation capacities in spinal cord (Decimo et al., 2011). In order to determine whether SC-LeSCs also react to mechanical injuries, we followed reponses to moderate contusive spinal cord injury (SCI). After 1, 3, 7 and 14 days of trauma, changes in histology, distribution, number, and proliferative capacities of nestin- and/or DCX-positive cells of the meninges were observed. Specifically, we observed a progressive increase in the thickness of the meninges close to the lesioned region, associated with an increase in the number of cells and a parallel, large and transient increase in the number of the nestin$^+$ proliferating (Ki67$^+$) cells (Decimo et al., 2011).

Not only the trauma induced proliferation of the nestin$^+$ population in meninges, but also induced a significant increase of DCX$^+$ cells, located adjacent to the basal lamina as well as in the thickness of the meninges, suggesting that the stem/ precursor cells present in meninges are activated by SCI. The meningeal responses were not limited to the site of the trauma and its surrounding area, in fact changes in nestin$^+$ cells also occurred in opposite (ventral) side, gradually spreading to the adjacent segments (Decimo et al., 2011).

To investigate if meningeal nestin$^+$ and DCX$^+$ cells may contribute to parenchymal reaction occurring during SCI, meningeal cells were transduced with a lentivirus expressin the fluorescent protein GFP (LV-GFP) 4 days before the injury. We observed that at 1, 7, and 30 days after SCI, the GFP-labelled cells were present in the core of fibrotic scar and were associated with fibronectin islands, in the glial scar, in the dorsal horn and in the perilesioned parenchyma. Most of the GFP$^+$ cells observed in the parenchyma outside the fibrotic area were nestin$^+$, and some of them express DCX (Decimo et al., 2011).

Thus, this work demonstrate that, following SCI, meningeal stem/ precursor cells proliferate, increase in number, and migrate in the parenchyma where they contribute to the parenchymal reaction. Altogether, the data suggest a possible significance of meninges as a niche for stem cells with functional implication in CNS physiopathology (Decimo et al., 2011).

Interestingly, this pattern of reactivity to injury (increased proliferation, expression of progenitor markers and migration) is a typical feature of the well-described neural stem cell niche of the SVZ (Bjornsson et al., 2015; Decimo et al., 2012b). Here, the niche shows a peculiar microenvironment that provides conditions for maintenance of the stem cell pools in a quiescent state as well as signals for activation and differentiation when neurogenesis is required (Bjornsson et al., 2015; Decimo et al., 2012a, 2012b; Scadden, 2006).

Almost in the same period, Nagakomi and colleagues demonstrate that meningeal pia mater cells have neural stem/ precursor cell (NSPC) activity in an adult mouse cortical infarction model, and that these cells in this particular environment can generate neurons (Nakagomi et al., 2011). Pia mater, close associated with blood vessels throughout the CNS, can be represented as perivascular cells/ pericytes, and these pericytes may be a potential sources of ischemia-induced NSPCs for neurogenesis following cortical infarction. Indeed, they found that NSCs migrated from the leptomeninges of post-stroke areas to the cortex through cortical layer 1, expressed NSPC markers such as nestin, formed neurosphere-like cell clusters with self-renewal activity, and differentiated into neurons, astrocytes, and oligodendrocytes (Nakagomi et al., 2011). Moreover, they expressed the marker DCX, demonstrating that meningeal-derived cells partecipate in CNS repair in poststroke cortex, as previously demonstrated in SCI (Decimo et al.,

2011; Nakagomi et al., 2012). In the poststroke brain, DCX^+ cells were observed within the post-stroke pia mater and cortex, where they appeared to localize near $nestin^+$ cells; however, they were not observed in the nonischemic ipsilateral pia mater and cortex, suggesting that ischemia triggers the induction of NSPCs and/or neuronal progenitors in these regions (Nakagomi et al., 2011, 2012, 2015a).

These results indicate that endogenous neurogenesis following traumatic injuries is a possible event, but it may have a limited therapeutic time window. It has been shown that, in fact, NSCs in post-stroke meninges and cortex peak for several days, but decline within a few weeks of stroke onset in both mice (Nakagomi et al., 2011) and humans (Nakayama et al., 2010).

$Nestin^+$ cells were found also in human brains of patients affected by meningiomas predominantly benign tumors of adults, usually attached to the dura mater (Petricevic et al., 2011). While in normal adult meninges, a moderate nestin expression was found in epithelial lining and within the subarachnoid space, in meningiomas expression of nestin was strongly detected in some part of the tumor, predominantly in the walls of blood vessels and in the surrounding tumor cells (Petricevic et al., 2011). The authors suggested that $nestin^+$ meningeal cells may give rise to tumor cells, independently of the tumor blood vessels. On the contrary, other authors described a similar pattern of nestin expression in tumor tissues, but suggested that the nestin expression is specific for proliferating endothelial cells exclusively (Sugawara et al., 2002). Thus, meningeal cells not only are injury-activated; they are also triggered by brain pathologies.

In a genetic mouse model of cerebellar ataxia (the Harlequin (Hq) mouse, Klein et al., 2002), the transplantation of human cerebellar granule neuron precursors (GNP) in the cerebellum triggered the proliferation of endogenous $nestin^+$ precursors in the leptomeninges that crossed the molecular layer and differentiated into mature neurons (Kumar et al., 2014). Indeed they found, 2 months after transplantation, a number of $Ki67^+$ cells, which increased significantly in GNP-treated animals. These data indicate that the cerebellar leptomeninges of Hq mice contain $nestin^+$ neural progenitors that are actively proliferating, meaning that endogenous neurogenesis is occuring in these regions. The cerebellar meninges of Hq mice have been demonstrated to be neurogenic *in vitro*, as they can form neurospheres and express MAP2 under differentiative conditions (Kumar et al.,

2014). Thus, the cerebellar leptomeninges of adult mice contain an endogenous neurogenic niche that can be stimulated to yield mature neurons from an not yet identified population of progenitors.

Overall, these works demonstrated that meninges are a novel NSC niche, hosting cells endowed, all over the CNS (brain, SC and cerebellum), of neural differentiation potential *in vitro* and after transplantation *in vivo*. In pathological conditions, as tumors, stroke or ataxia models, and traumatic injury like SCI, meninges display an intense response to neuronal degeneration and active parenchymal reaction probably finalized to tissue repair. However, the neurogenic potential of meningeal NPs in physiological conditions has never been investigated.

AIMS

During recent years, embryonic and adult neural stem cells (NSCs) attracted attention from the scientific community as major candidates for regenerative and cell replacement therapies. The clinical application of adult NSCs, despite their potentially useful properties of self-renewal, neuro-glia differentiation potential and their possible use for transplantation in the autologous setting, is still of debate. Among technical concerns, of relevance is that site of sampling NSCs are hardly accessible, are difficult to expand *in vitro* as homogeneous stem cell population and show low *in vivo* neuronal differentiation efficiency.

Thanks to their superficial location, meninges may represent a new easily accessible tissue for collection of NSCs for regenerative medicine and autologous transplant.

During the course of my studies, I explored the hypothesis that meningeal NPs may contribute to neurogenesis during postnatal development. In order to demonstrate this hypothesis, I focused on the following aims (Figure 1.18):

Aim 1. Genetically label and visualize meningeal NPs and their progeny.

To the best of our knowledge, no transgenic model exclusively labelling the meningeal tissue is available. Therefore, the bottleneck of this project was represented by the need to find a technique to label permanently only the cells residing in the meninges. To solve this issue, I approached several labelling techniques and tested different methods to visualize and follow the meningeal cells during the postnatal development, including lentiviral transduction, plasmid electroporation and dyes injection.

Aim 2. Evaluate the migratory pathway of meningeal NPs from their meningeal location to the brain parenchyma.

Once developed a reliable and reproducible technique to label exclusively meningeal cells, I focused on the investigation of meningeal cells migratory potential. I thus performed time course analysis and time-lapse movies to determine how meningeal

cells migrate to the cortex, and which path do they follow to reach their finally location.

Aim 3. Determine the fate of meningeal NPs migrated in the brain parenchyma.

In order to define the differentiation potential of meningeal- derived migrated cells, I characterized the phenotype of the cells later in development, once they've reached their final location in the brain parenchyma; I then investigated the functionality of these cells and their integration in the local network by electrophysiological experiments.

Aim 4. Determine the identity of meningeal NPs migrated in the brain parenchyma.

In this section, I investigated the identity of migrated meningeal cells, taking advantage of the new technologies in the transgenesis field. Indeed, I performed lineage-tracing experiments using different transgenic mouse lines to visualize the specific populations, *i.e.* radial glial population GLAST and Nestin, and perivascular PDGFRβ population, to study the population of origin of meningeal cells that migrated into the brain parenchyma. Moreover, by means of EdU injection, I studied the embryonic derivation of these cells.

Aim 5. Determine the developmental origin of meningeal NPs migrated in the brain parenchyma.

Finally, in order to investigate the developmental origin of meningeal-derived cells, I performed some pilot lineage-tracing experiments, analysing the Wnt1 population, to identify a possible neural crest developmental origin of meningeal derived cells based on the known neural crest derivation of forebrain meninges.

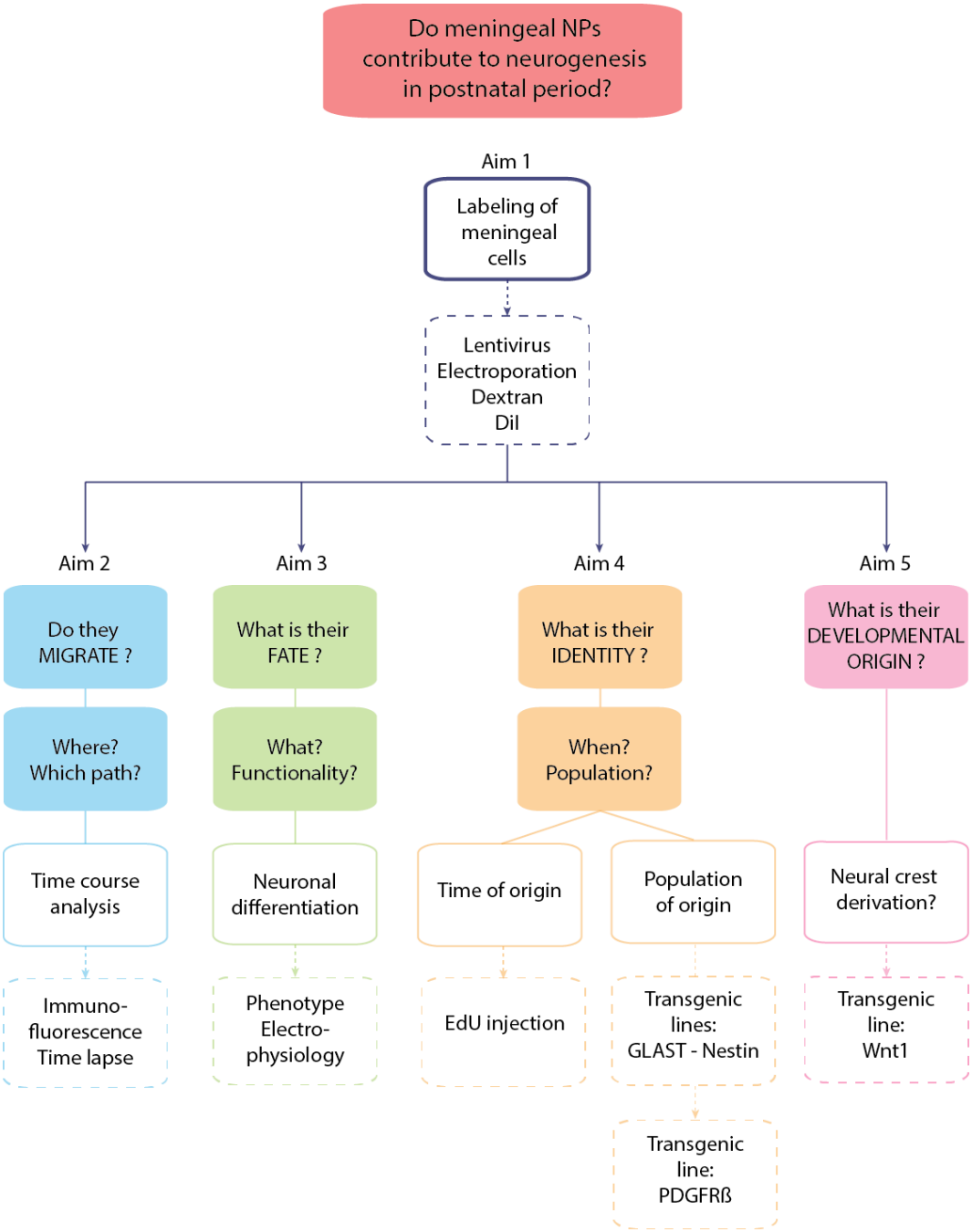

Figure 1.18. Experimental plan.

The experimental plan summarizes the objectives of this project. First, we genetically labelled meningeal NPs (blue) in order to follow them and their progeny, then we studied the migration potential and the migratory path (light blue) that meningeal NPs followed to reach their final location in the cortex. We determined the phenotype and the functionality (green) of meningeal-derived cells and we explored the time and population of origin (orange) of these cells. Finally, we will explore the developmental origin of meningeal-derived cells (pink).

CHAPTER 2

MATERIAL AND METHODS

2.1 - Animals

Animal housing and all experimental procedures were approved by the Animal Ethics Committee of the KU Leuven (Belgium). Wild type (WT) CD1 mice and C57/BL6 mice were obtained from the university animal facility. The following transgenic mouse lines were used: Wnt1-Cre mice constitutively expressing Cre recombinase under the control of the Wnt1 promoter (Jiang et al., 2002); PDGFRß-Cre mice constitutively expressing Cre recombinase under the control of the PDGFRß promoter (Foo et al., 2006); GLAST-Cre^{ERT2} mice expressing tamoxifen inducible Cre under the control of the GLAST promoter (Mori et al., 2006); Nestin-Cre^{ERT2} mice expressing tamoxifen inducible Cre under the control of the Nestin promoter (Lagace et al., 2007); Rosa26-lox-stop-lox-YFP reporter mice expressing YFP after Cre-mediated lox recombination (Srinivas et al., 2001). All these lines were on a pure C57/BL6 genetic background. The Wnt1-Cre, PDGFRß-Cre, GLAST-Cre^{ERT2}, Nestin-Cre^{ERT2} driver lines were intercrossed with the Rosa26-lox-stop-lox-YFP reporter mouse line, thus resulting in the lines named Wnt1-YFP, PDGFRß-YFP, GLAST-YFP and Nestin-YFP, respectively. Cre-mediated recombination of the lox-stop-lox cassette in these lines results in permanent YFP expression by the cells, expressing the Cre-driver. In these studies, presence of a vaginal plug determined the embryonic day (E) 0.5 and the day of birth is designated as postnatal day (P) 0. Cre-mediated recombination in Cre^{ERT2} transgenic mice was induced by administration of Tamoxifen (Sigma), dissolved at 30 mg/ml in sunflower oil (Sigma). A single dose of 3 mg of Tamoxifen was administered via gavage to pregnant GLAST-YFP and Nestin-YFP mice at E13.5.

2.2 - Lentiviral production

Lentiviral vector expressing CherryRed, plvx-GFP or Brainbow1.0(L) was used. Production of lentiviruses was performed by plasmid transfection into HEK-293T cells as described (Carlotti et al., 2004). The titer of the lentiviral vector preparations

was 1-4x10^9 transducing units/ml. Before injection, lentiviral suspension was mixed with 0.05% FastGreen 1:10 (Sigma).

2.3 - Plasmid preparation

The following expression plasmids were used for electroporation: pCAGGS-mRFP (kindly provided by Dr. Calegari F., CRTD, Dresden, Germany), Brainbow1.0(L) (kindly provided by Dr. Livet J., Inserm U592-UPMC, Paris, France) and pOG231 (expressing NLS-Cre recombinase; kindly provided by Dr. O'Gorman S., Salk Institute for Biological Studies, La Jolla, USA). DNA was prepared using the PureLink® HiPure Plasmid Maxiprep Kit (Invitrogen), purified with EndoFree kit (Qiagen) and resuspended in sterile PBS to a final concentration of 3-5 μg/μl. Plasmids were then mixed with 0.05% FastGreen 1:10 (Sigma) and the mixture was centrifuged for 30 seconds at 16'000 g to remove precipitates.

2.4 - Production of fluorescent lentiviral particles

To obtained fluorescent lentiviral particles, HEK-293T cells were stained with the fluorescent cell dye Carboxyfluorescein succinimidyl ester (CFSE; Life Technologies) (Fig. S1B). CFSE readily diffuses into cells where intracellular esterases cleave its acetate groups, turning the colorless CFSE into a highly fluorescent amine-reactive 5(6)-CFSE (equivalent to Fluorescein). Fluorescent amine-reactive 5(6)-CFSE spontaneously and irreversibly couples to both intracellular and cell-surface proteins by reaction with lysine side chains and other available amine groups resulting in permanent dye–protein adducts.

Briefly, HEK-293T were centrifuged (300 g, 5 minutes), the cellular pellet was resuspended in pre-warmed (37°C) PBS/ 0.1% bovine albumin serum (BSA) containing CFSE (20 μM) and incubated for 15 minutes at 37°C. Thereafter, the cell suspension was quenched with ice could DMEM supplemented with 10% fetal bovine serum (FBS) and incubated 5 minutes on ice. HEK-293T cells were centrifuge (300 g, 5 minutes), re-washed for 3 times and plated in DMEM supplemented with 10% FBS. Production of lentiviruses was performed by plasmid transfection into HEK-293T cells as described (Carlotti et al., 2004). Lentiviral particles fluorescence intensity (excitation/emission maxima ~495/525 nm) were checked by confocal microscopy (Fig. S1B,C). The titer of the lentiviral vector

preparations was 1-4x10^9 transducing units/ml. Before injection, the lentiviral suspension was mixed with 0.05% FastGreen 1:10 (Sigma). Distribution of the fluorescent lentiviral particles was assessed after 1 hour from lentiviral meningeal injection (Fig. S1D) using a Leica DMI6000 B fluorescence microscope (Leica Microsystems, Mannheim, Germany).

2.5 - *In vivo* cell labelling techniques

WT and transgenic P0/P1 pups were anesthetized by hypothermia. Lentiviral suspension (1-4x10^9 transducing units/ml), plasmid suspension (3.0 μg/μl), dextran solution (FITC-conjugated, 500kDa, 5 μg/ml in physiological solution, 0.9% NaCl, SIGMA), or DiI solution (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate, 20 μg/ml in ethanol, Life Technologies) were loaded into a previously pulled borosilicate glass capillary. The skull was carefully pierced and 1-2 μl of each solution was pressure-injected into the subarachnoid region, cisterna magna or ventricle using a FemtoJet injector (Eppendorf).

For plasmid electroporation, in order to generate an electric field allowing the negatively charged plasmid DNA transfecting only the meninges and not the cortex, electrodes were placed with the plus pole at the surface of the skull. Platinum tweezertrodes were then used to electroporate with five pulses of 135 V (50 ms; separated by 950 ms), generated using the ECM 830 BTX Electroporator (Harvard Apparatus). SignaGel was used to increase conductance.

2.6 - RNA analysis

RNA expression analysis was performed by Taqman quantitative RT-PCR as described (Carmeliet et al., 2001), using in house-designed primers and probes or premade primer sets (Applied Biosystems, Carlsbad, CA).

2.7 - Cerebrospinal fluid sampling and Elisa

WT CD1 P0 pups were anesthetized by hypothermia and lentiviral vector expressing CherryRed was injected in the meninges as described above, or intrathecally as control. After 2 and 12 hours post injection of the viral vector, we sampled CSF by direct extraction from the cisterna magna. Collected CSF was

maintained ice-cold and ELISA analysis for the viral protein p24 was performed as previously described (Carmeliet et al., 1998).

2.8 - Tissue preparation for live imaging

WT CD1 P0 pups were anesthetized by hypothermia and DiI solution was injected in the meninges as described above. At 24 and 72 hours after meningeal injection, mice were anesthetized, decapitated and brains were collected in ice-cold artificial cerebro-spinal fluid (ACSF) (KCl 2.5 mM, MgCl $6H_2O$ 1mM, NaH_2PO_4 1.25 mM, $CaCl_2$ $2H_2O$ 2mM, $NaHCO_3$ 25mM, D-glucose 25 mM, sucrose 210.5 mM). Sagittal brain tissue sections (200 µm thick) were cut with a Microm HM 650V vibratome (Fisher Scientific UK Ltd, Loughborough, UK), collected in Neurobasal medium (Gibco) without growth factors, and equilibrated at 37°C, 5% CO_2 for at least 1 hour before the imaging. After the imaging, the motility of the cells was analysed using the Tracking Tool Software (Gradientech) by tracking nucleus position over time and by superimposing migration origin at the zero-cross point.

2.9 - Immunohistochemistry

2.9.1 - Tissue preparation

For post hoc *in vivo* studies, mice were anesthetized by intraperitoneal injection with Nembutal (1:10 in physiological solution, 0.9% NaCl) and were sacrificed by intracardial perfusion of PBS containing 4% paraformaldehyde (PFA) / 4% sucrose (pH 7.4) solution. Brains were extracted from the skull and post-fixed overnight in 4% PFA / 4% sucrose at 4°C, then rinsed in PBS and equilibrated and stored in sucrose 30% at 4°C. For mice younger than P5, the brains were directly fixed for 2 days in 4% PFA / 4% sucrose without prior perfusion, then rinsed in PBS and equilibrated and stored in sucrose 30% at 4°C. Samples were cryosectioned at 30 µm, in sagittal sections. Slides were stored at -20°C. Immunostaining on cryosections was performed after 30 minutes incubation in blocking solution 1 (PBS with 0.25% Triton X-100, 2% BSA and 1% FBS). Sections were then incubated with primary antibodies in blocking solution 1 overnight at 4°C. After rinsing 6 times for 5 minutes in blocking solution 1, appropriate secondary antibodies were applied for 4 hours at room temperature. If needed, after rinsing in blocking solution 2 (PBS with

0.25% Triton X-100, 2% BSA and 5% rabbit serum), pre-conjugated antibody (Alexa Fluor 488 conjugated anti-GFP) was incubated overnight at 4°C. After final washing steps in PBS, nuclear staining with 4',6-Diamidino-2-Phenylindole Dihydrochloride (DAPI, Life Technologies) was performed and slides were mounted using ProLong® Gold antifade reagent (Life Technologies). The stained slides were store at 4°C. H&E or Harris Haematoxylin staining staining of frozen sections was done using standard protocols. For whole-mount staining of 100 μm thick sections, fixed samples were sectioned with a Microm HM 650V vibratome (Fisher Scientific UK Ltd, Loughborough, UK) in sagittal plane and stored in PBS at 4°C. Immunostaining was performed after 30 minutes incubation in blocking solution (PBS with 0.1% Triton X-100 and 10% FBS). Sections were then incubated with primary antibodies in blocking solution for 60-72 hours at 4°C. After rinsing 6 times for 5 minutes in blocking solution, appropriate secondary antibodies were applied overnight at 4°C. If needed, after rinsing in blocking solution, pre-conjugated antibody was incubated overnight at 4°C. After final washing steps in PBS, nuclear staining with DAPI (Life Technologies) was performed and slides were mounted using ProLong® Gold antifade reagent (Life Technologies). The stained slides were stored at 4°C.

2.9.2 - Antibodies

The following primary antibodies were used: anti-GFP (rabbit, 1:200, Molecular Probes, A11122), anti-PDGFRβ (goat, 1:200, R&D, AF1042), anti-RFP (rabbit, 1:200, Rockland, 600-401-379), anti-NeuN (mouse, 1:100, Millipore, MAB377), anti-Satb2 (rabbit, 1:200, abcam, ab34735), anti-GAD65/67 (rabbit, 1:200, Millipore, AB1511), anti-NG2 (rabbit, 1:200, Millipore, AB5320), anti-Glast (anti-EAAT1; rabbit, 1:200, abcam, AB416), anti-HuC/HuD (mouse, 1:100, Invitrogen, A-21271), anti-laminin (rabbit, 1:400, Sigma, L9393), anti-GFAP (rabbit, 1:200, DAKO, Z033401), anti-GFAP (mouse, 1:200, Sigma, g3893) anti-Blbp (rabbit, 1:100, abcam, ab32423), anti-DCX (goat, 1:200, Santa Cruz, sc-8066), anti-Iba1 (rabbit, 1:400, WAKO, 019-19741). A primary antibody Alexa Fluor 488-conjugated anti-GFP antibody was also used (rabbit, 1:100, Molecular Probes, A21311). The following secondary antibodies were used: donkey anti-rabbit Alexa Fluor 488, donkey anti-rabbit Alexa Fluor 568, donkey anti-rabbit Alexa Fluor 647, donkey anti-mouse Alexa

Fluor 647, donkey anti-goat Alexa Fluor 568 (1:500, all from Molecular Probes), donkey anti-rabbit CY3 (1:1000, Jackson), donkey anti-mouse CY3 (1:500, Jackson).

2.9.3 - Imaging and quantification

For analyses on *post hoc* tissue, imaging of the immunostained slices was performed using a Leica DMI6000 B epifluorescence microscope (Leica Microsystems, Mannheim, Germany), Zeiss LSM 510 Meta NLO or Zeiss LSM 780 confocal microscope (oil objectives: x 40 with NA 1.3, x 63 with NA 1.4, x 100 with NA 1.46) (Carl Zeiss, Munich, Germany). For 3D reconstruction, confocal images were deconvoluted with Huygens Professional software (Scientific Volume Imaging B.V., Hilversum, The Netherlands) and 4D animations of the deconvoluted images were created with Imaris 7.7.1 software (Bitplane AG, Zurich, Switzerland).

CherryRed expressing cells were visualized by immunostaining with anti-RFP antibody. YFP expressing cells in PDGFRß-YFP, GLAST-YFP and Nestin-YFP animals were visualized by immunostaining with anti-GFP. Brainbow 1.0(L) expressing cells were visualized by immunostaining with anti-RFP and anti-GFP (CFP and YFP) antibodies (see "Antibodies" section above).

Quantifications of the double positive immunostained cells and of the recombined cells in the mice injected with lentiviral vector expressing the Brainbow1.0(L) reporter were performed on brain sagittal sections from at least $n \geq 3$ mice. We considered cells as double positive when the signal of the fluorescent protein co-localized with the marker present in the nucleus (NeuN, Satb2), perinuclear cytosol (GAD65/67) or cytosol (perinuclear and processes) (GFAP, NG2, Iba1) on a single optical section. DAPI was used to visualize the cell nuclei. A mean of $n = 164.8 \pm 97.5$ cells were counted for each marker for each animal analysed (total cells counted = 12,858).

2.10 - EdU administration

For birthdating experiments, EdU dissolved in 0.9% NaCl, was administered with a single intraperitoneal injection to pregnant WT mice from E12 to E18. The dose of EdU was settled at 10 mg/kg bodyweight, in order to achieve readily detectable EdU incorporation (Chehrehasa et al., 2009).

2.11 – Electrophysiology

In collaboration with Prof. Michele Giugliano and Dr. Gabriella Panuccio, Theoretical Neurobiology & Neuroengineering Lab, University of Antwerp (Belgium)

2.11.1 - Brain slices preparation

Acute cortical slices for electrophysiology experiments were obtained from P21-P30 CD1 mice injected at P0 in their meninges with a lentiviral vector expressing CherryRed. Animals were deeply anaesthetized (5% isoflurane, 500-1000ml/min) and decapitated. The brain was rapidly extracted and placed in ice-cold "cutting" ACSF, containing: KCl 2.5 mM, $MgCl_2$ 7 mM, NaH_2PO_4 1.25 mM, $CaCl_2$ 0.5 mM, $NaHCO_3$ 25 mM, D-glucose 25 mM, sucrose 183 mM, L-Ascorbic Acid 1 mM, Na^+-Pyruvate 3 mM (315 mosm/Kg, pH 7.4 when bubbled with carbogen, i.e., 95% O_2 5% CO_2). Coronal brain tissue sections (200 mm thick) comprising the primary somatosensory cortex (S1) were cut with a vibratome (VT1000s, Leica Instruments, Nussloch, Germany or 7000 smz 2, Campden Instruments, Loughborough, UK) in ice-cold cutting ACSF, and then stored at room temperature in "standard" ASCF, containing: NaCl 125 mM, KCl 2.5 mM, $MgCl_2$ 1 mM, NaH_2PO_4 1.25 mM, $CaCl_2$ 2 mM, $NaHCO_3$ 25 mM, D-glucose 25 mM, L-Ascorbic Acid 1 mM (315 mosm/Kg, pH 7.4 when bubbled with carbogen, i.e., 95% O_2 5% CO_2). Slices were let recover for at least 60 minutes prior to electrophysiological recordings and remained viable for up to 8 hours. All chemicals were obtained from Sigma, Belgium.

2.11.2 - Patch-clamp recordings

Individual slices were transferred to a (submerged) recording chamber, replacing the stage of a microscope. Electrophysiological characterization of CherryRed cells and control cortical neurons was performed employing the patch-clamp technique in whole-cell configuration (Hamill et al., 1981). Recordings were performed as in (Köndgen et al., 2008) at 32 - 34°C, under constant perfusion of "standard" ACSF (1.5 ml/min). Cell visualization and patch pipette micromanipulation were performed by videomicroscopy, employing a 40x water-immersion objective mounted on an upright microscope equipped with infrared differential interference contrast (IR-DIC) and epifluorescence optics (SliceScope, Scientifica, Uckfield, UK). Perivascular meningeal derived cells were identified under epifluorescence by their CherryRed tags (Fig.2S), and were localized mainly in upper cortical layers I-IV. Somatic patch

recordings were carried out in both CherryRed-positive cells and in neighboring CherryRed-negative control neurons, employing glass pipette electrodes pulled from borosilicate capillaries (GC150-10, Harvard Apparatus, Holliston, MA, USA) using a P-97 horizontal puller (Sutter Instruments, Novato, CA, USA). Electrodes had tip diameters of ~2 μm and resistance of 3-5 MΩ when filled with an intracellular solution containing : K^+-gluconate 135 mM, NaCl 4 mM, HEPES 10 mM, EGTA 0.2 mM, ATP-Mg^{2+} 2 mM, GTP-Na_2 0.3 mM, phospho-creatine-Na_2 10 mM and biocytin 0.2% (290 mosm/Kg, pH 7.3 adjusted with KOH). Intracellular membrane electrical potentials were recorded in current-clamp mode, using a Multiclamp 700B amplifier (Molecular Devices, Palo Alto, CA, USA), sampled at 20kHz and digitized using a 16 bits DAQ board (NI SSC-68, National Instruments, Austin, TX, USA), employing the software LCG (Linaro et al., 2014). The same software was also used to synthesize stimulus waveforms. Hardware bridge-balance and capacitance compensation circuitry were not used, while non-parametric compensation of electrode artifacts was employed off-line via LCG (Brette et al., 2008).

2.12 - Statistics

Recorded voltage traces were analysed by custom MATLAB scripts (The MathWorks, Natick, MA, USA). Data are expressed as mean ± SD, while in the graphs the error bars are expressing mean ± SEM. Statistical differences were calculated by two-tailed unpaired t-test for two data sets and ANOVA followed by Bonferroni post-hoc test for multiple data sets using Prism (GraphPad Inc.). $P < 0.05$ was considered statistically significant.

CHAPTER 3

RESULTS

3.1 – Specific labelling of meningeal cells

In this study, we explored the hypothesis that progenitors in meninges and meningeal substructures located outside the brain parenchyma, differentiate *in vivo* into neural cells, without further proliferation. Since a transgenic driver that selectively labels meningeal cells is not available for lineage tracing, we developed a tecnique to exclusively label meningeal cells with different cell tracers.

We decided to transduce meningeal cells with a lentiviral vector (LV) expressing a fluorescent protein, either GFP or CherryRed (LV-GFP or LV-CherryRed, respectively). To this aim, a LV-CherryRed solution was loaded into a borosilicate glass capillary and pressure-injected the solution in the meninges of the right hemisphere of newborn (P0) pups, specifically in the posterior region of the cerebral cortex taking as reference the position of the lambda, where parietal bones join the occipital bone (Figure 3.1).

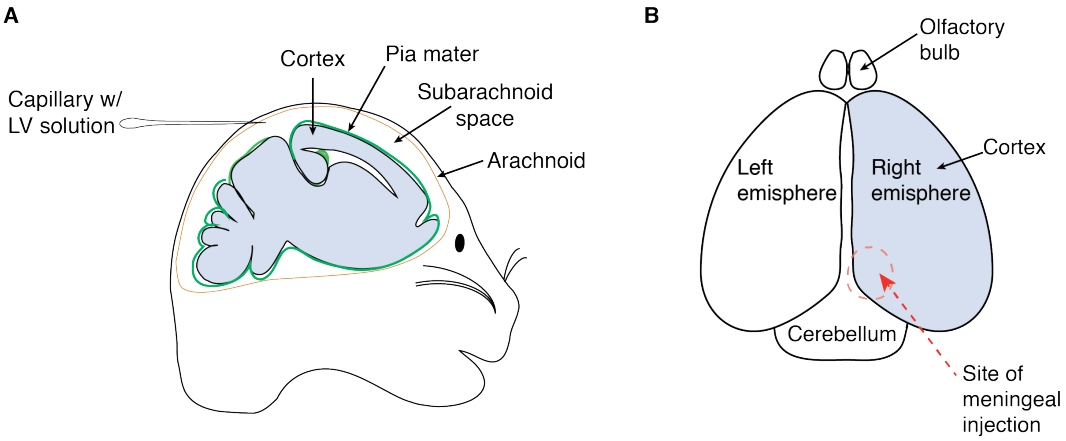

Figure 3.1. Schematic representation of the injection in the meninges.

A, Sagittal view of the pressure-injection of LV in the subarachnoid space, between the outer layer (arachnoid; orange line) and inner layer (pia mater; green line) of the meninges. **B,** Frontal view of the site of meningeal injection, located posteriorly at the level of the lambda, where the parietal bones join the occipital bone (red dashed lines).

The LV-fluorescent protein expression was detectable 15 hours after the injection. At this time point, we analysed the location of CherryRed^{+} cells throughout the entire brain, along the rostro-caudal, medio-lateral and dorso-ventral axes. We detected CherryRed^{+} cells in the meningeal tissue only, and no labelling of other structures, as cortex, VZ or any other regions of the brain (Figure 3.2).

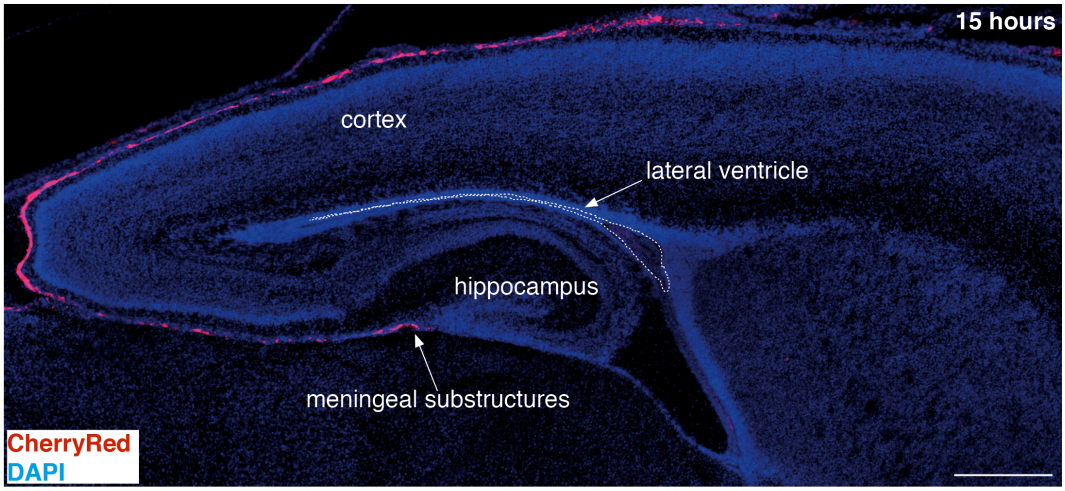

Figure 3.2. Specific meningeal labelling by means of LV-CherryRed injection in the meninges.

Injection of a LV-CherryRed in the meninges of a P0 CD1 mouse and analysis 15 hours later revealed the presence of labelled CherryRed^{+} cells in the meningeal tissue only. The dashed line delineates the ventricle. Scale bar: 200 μm.

Since the expression of the LV-fluorescent protein was not detectable at time points earlier than ~15 hours after the LV injection in the meninges, we verified the specifity of LV labelling soon after the injection by co-injecting the LV with a fluorescent dextran dye (500 kDa, Figure 3.3) that is immediately visible after the injection. Analysis of the entire brain sections confirmed that the dextran dye was confined to the meninges and meningeal substructures early after injection (~1 hour), and was undetectable in the cortical parenchyma, ventricles or elsewhere in the brain (Figure 3.3).

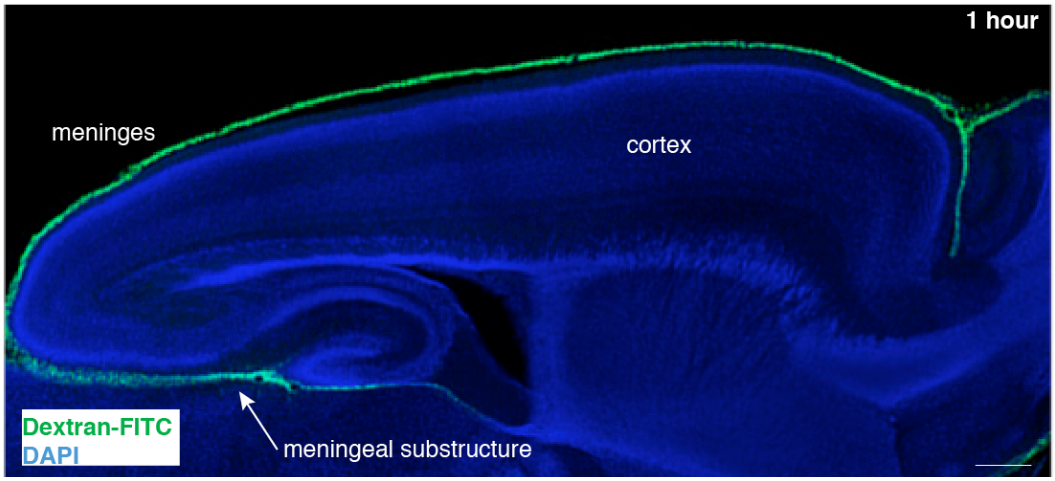

Figure 3.3. Sagittal brain section of a P0 CD1 mouse injected in the meninges with FITC-dextran dye (500 kDa).

Figure shows that at 1 hour post meningeal injection, the dye (green) is confined to the meninges and meningeal substructures and undetectable in the cortical parenchyma, ventricles, or elsewhere in the brain. Scale bar: 200 μm.

The result of the meninges-restricted distribution of dextran strongly support the hypothesis that, when injected in the meninges at P0, LV remained confined to the meninges since the first time point after the injection (Figure 3.3, 3.4).

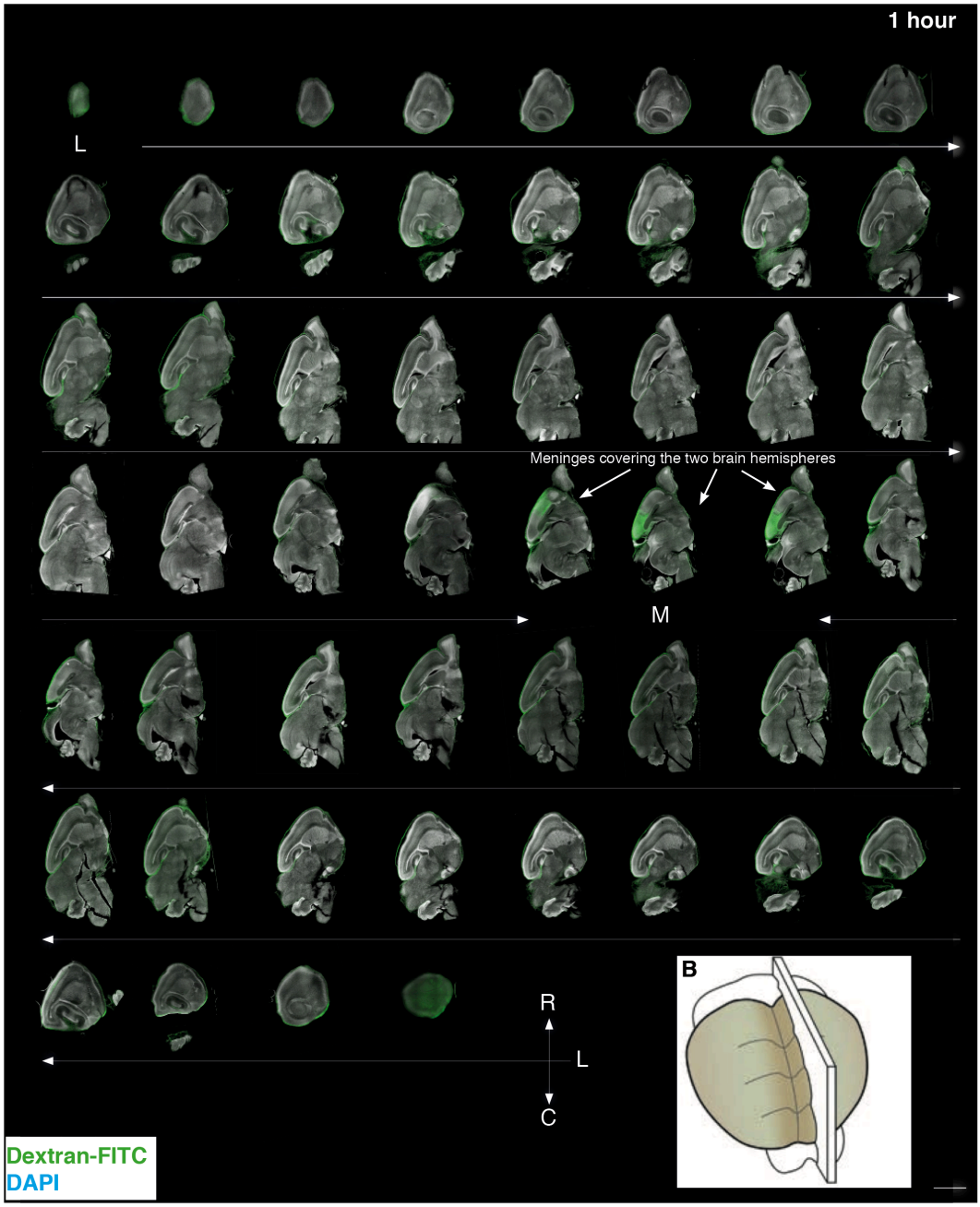

Figure 3.4. Serial sagittal sections through the entire brain of a P0 CD1 mouse injected in the meninges with dextran dye (500 kDa).

A,B, Serial sagittal sections through the entire brain of a P0 C57/BL6 neonate injected in the meninges with dextran dye (500 kDa) showing that at 1 hour post meningeal injection, the dye (green) is confined to the meninges and meningeal substructures and undetectable in the cortical parenchyma, ventricles or elsewhere in the brain parenchyma. In parasagittal medial sections (M), the green signal is prominently visible in the meninges covering the hemisphere at the medial portion of the brain, where both hemispheres curve towards the interhemispheric fissure (see scheme in panel B). Horizontal arrows indicate the progression from brain lateral sections (L) to medial (M) while the vertical arrows

indicate rostro (R) caudal (C) brain axis. To visualize the thin green meninges on the surface of the brain, an image showing only the meninges is shown side-by-side. Scale bar: 1 mm.

To support this information, we devised a technique to directly visualize the distribution of the injected LV-particles. To this aim, we produced fluorescently labelled LV-particles (CSFE-LV, see Material and Methods, 2.4; Figure 3.5A,B), by adding Carboxyfluorescein succinimidyl ester (CSFE), a fluorescent cell dye, to HEK-293T cell suspension used for the production of LV-particles. CSFE spontaneously and irreversibly couples to both intracellular and cell-surface proteins of HEK-293T cells. Production of LV-Cherry particles was then performed by plasmid transfection into CSFE-labelled HEK-293T cells, obtaining CSFE-labelled LV-particles.

We then injected CFSE-LV-particles in the meninges of P0 mice. We were able to detect CFSE-labelled cells immediately (~1 hour) after meningeal transduction, and we confirmed that $CFSE^{+}$ cells were confined in the meninges and meningeal substructures, without any labelling of the cortical parenchyma, or any other region of the brain (Figure 3.5C).

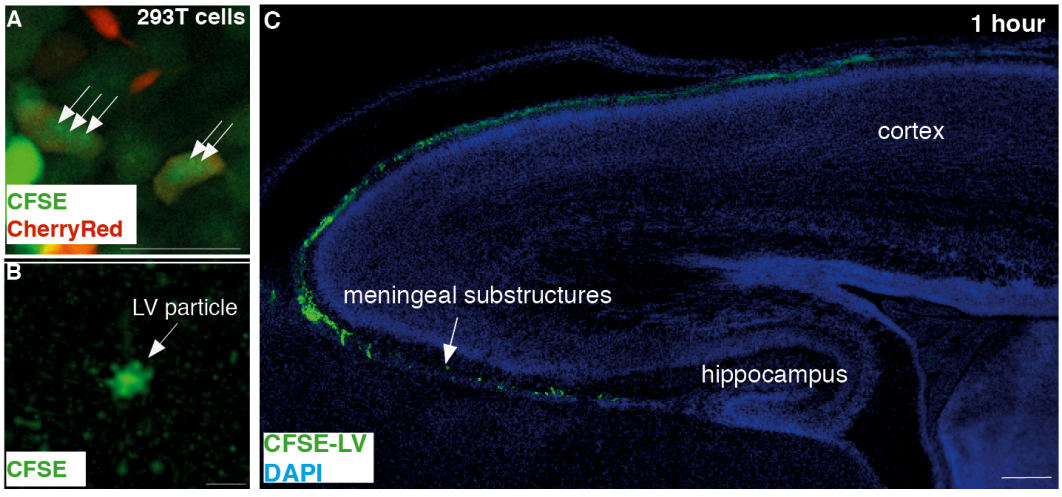

Figure 3.5. Sagittal brain section of a P0 CD1 mouse injected in the meninges with the CFSE-labelled LV-CherryRed.

A, HEK-293T cells incubated with the vital fluorescent cell dye CFSE (green) and transfected with the plasmid for CherryRed (red) LV production. Arrows point to budding CFSE labelled-LV-particles. **B,** Confocal microscopy showing the presence of CFSE fluorescent LV-particles (green, arrow) in the LV suspension before the meningeal injection. **C,** At 1 hour post meningeal injection,

the CFSE$^+$ signal (green) is confined to the meninges and meningeal substructures and undetectable in the cortical parenchyma. Scale bar: 20 µm (A), 0.2 µm (B), 200 µm (C).

Altogether, our data suggest that LV injection into the meninges of P0 pups allows the specific labelling of meningeal cells only.

To further validate our technique, we labelled the meningeal cells by plasmid electroporation of an RFP-encoding plasmid in the meninges of P0 mice (Figure 3.6A). RFP$^+$ labelled cells were detectable at early time points (~8 hours), and they were confined in the meninges and meningeal substructures, confirming that our meningeal labelling technique is specific and exclusive for meningeal cells. Importantly, the transfection protocol used ensured that the plasmid DNA selectively transfected meningeal and not brain parenchymal cells. Indeed, the electric field was oriented in such a way that the plasmid DNA entered meningeal but not brain cortical cells (Figure 3.6B).

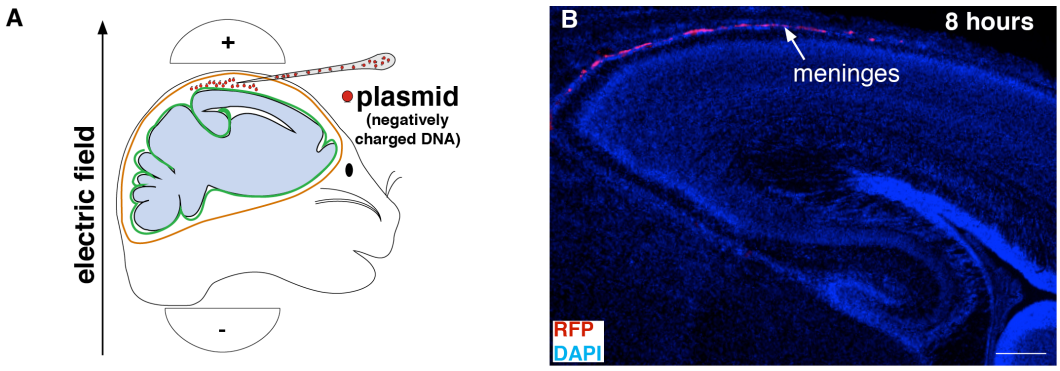

Figure 3.6. Electroporation of the meninges with an RFP encoding plasmid P0 C57/BL6 mouse.

A, Schematic representation of the electroporation of an RFP encoding plasmid (denoted by red dots) in the brain meninges after pressure-injection of the DNA into the subarachnoid space between the outer layer (red/brown line) and inner layer (pia mater; green line) of the meninges. The electric field is oriented with the plus pole on the surface of the skull in order to transfect selectively the meningeal but not the parenchymal cells with the negatively charged DNA. **B,** Sagittal brain section of P0 C57/BL6 mice after electroporation of the meninges with an RFP encoding plasmid, showing RFP$^+$ cells in the meninges at 8 hours after electroporation. Scale bar: 200 µm.

For conclusive confirmation, we labelled meningeal cells with another technique, entirely independent of LV transduction or plasmid transfection and based onm the

use of DiI, a fluorescent carbocyanine-type dye that labels the cellular plasma membrane because of its lipophilic nature. We injected the DiI in the meninges of P0 pups and checked the distribution of DiI-labelled cells in the brain cortex at early time points (1 hours, Figure 3.7A). We observed that the DiI^+ cells were confined to the meninges only, and were not detected in the brain parenchyma (cortex) or VZ (Figure 3.7B).

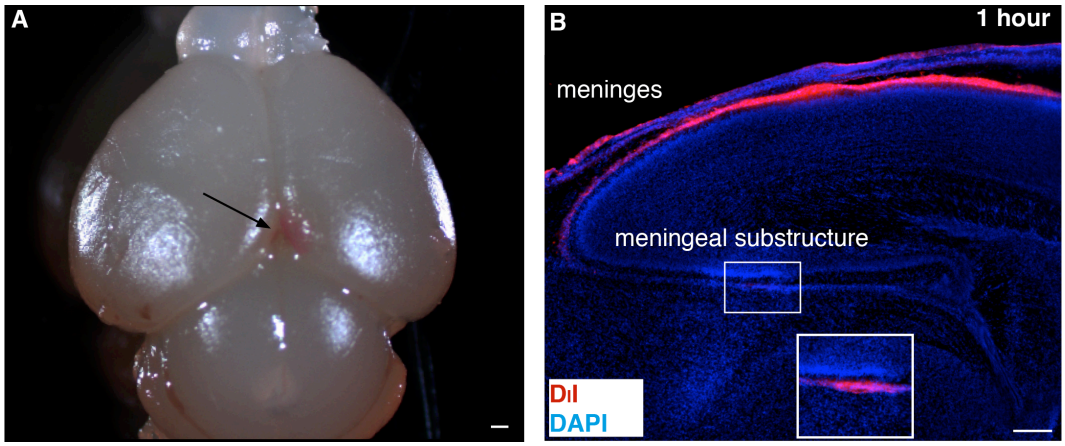

Figure 3.7. Meningeal injection of DiI in P0 CD1 pups.

A, Macroscopic appearance of the whole brain injected with DiI (red, arrow) in the meninges. **B,** At 1 hour after meningeal injection, DiI^+ cells were confined to the meninges and meningeal substructures and undetectable in the cortical parenchyma. The inset in B represents a higher magnification of the boxed area. Scale bars: 1 mm (A), 200 μm (B).

Overall, our data suggest that we developed a panel of reliable and reproducible techniques that allow the specific labelling of meningeal cells. Indeed, we confirmed that we are able to target exclusively meninges by injection of lentiviruses, dextran, fluorescently labelled LV particles, DiI, and plasmid electroporation in P0 mouse brain meninges. The reproducibility of the results obtained with all the mentioned tracers demonstrates the strength of our labelling technique and thus allows cell fate/ tracing studies.

3.2 – Meningeal cells migrate in the postnatal cortex

3.2.1 – Time course analysis: meningeal labelling by LV-transduction

To explore the possibility that cells residing in the meninges were able to migrate and differentiate into neural cells in postnatal mouse brain *in vivo*, we performed a time-course analysis of LV-labelled meningeal cells.

We analysed the anatomical distribution of CherryRed$^+$ cells at 15 and 24-48 hours, and at 3-5 days after transduction of the meningeal cells with LV-CherryRed in P0 pups. At 15 hours, 100% of the CherryRed$^+$ cells were confined to the meninges and meningeal substructures in the brain (Figure 3.8A-C, Figure 3.9); by 24-48 hours, 14.6% of the CherryRed$^+$ cells were detected in the dorso-caudal VZ and SVZ, and 10.4% was detected in the lower layers of the cortex (Figure 8D-F, Figure 3.9). At 3-5 days, ~53% of the CherryRed$^+$ cells were distributed in the VZ and the lower cortical layer VI (Figure 3.8G-I, 3.9).

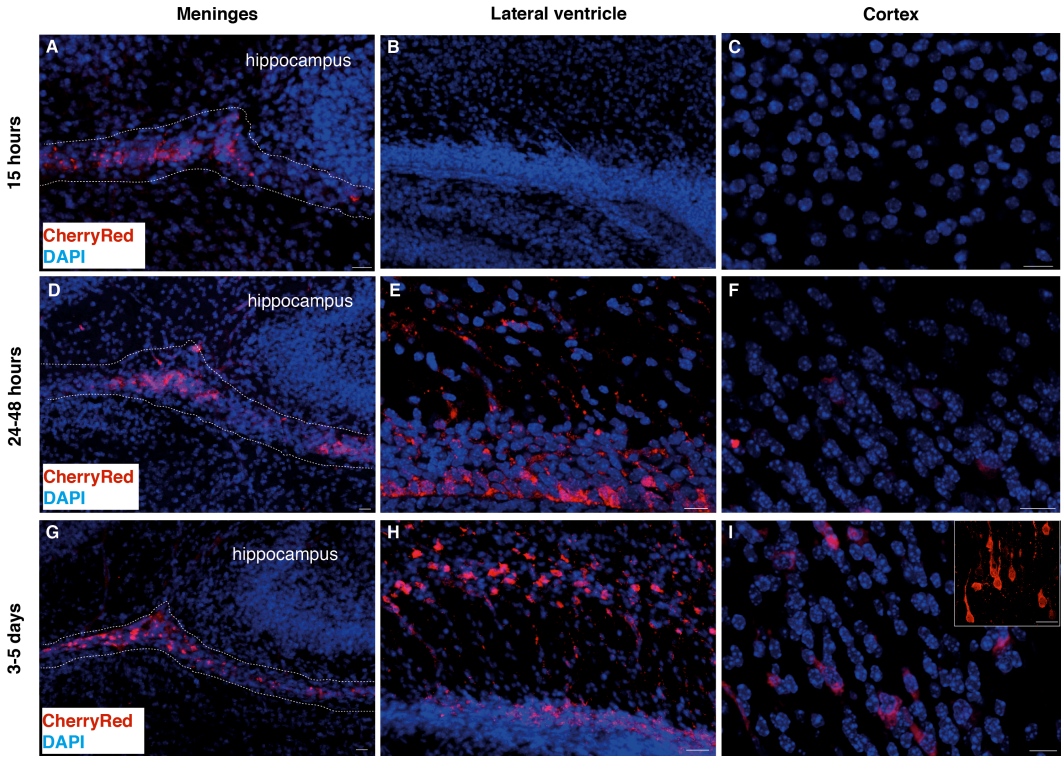

Figure 3.8. Time course analysis of sagittal brain sections of CD1 mice injected in the meninges with the lentiviral vector expressing CherryRed at P0.

Distribution of CherryRed$^+$ cells is shown after 15 (A-C), 24-48 hours (D-F) and 3-5 days (G-I) after lentiviral injection. Dashed line delineates the meningeal substructure.

A-C, At 15 hours after meningeal transduction, CherryRed$^+$ cells were confined to the meninges and meningeal substructures (A) and undetectable in the cortical parenchyma (C), ventricles (B), or elsewhere in the brain. **D-F,** By 24-48 hours after meningeal injection, CherryRed$^+$ cells migrated to the ventricular and subventricular zone (E). Labelled cells are still present in the meningeal tissue (D), and a minor proportion is already visible in the lower layers of the cortex (F). **G-I,** By 3-4 days after injection, LV-Cherry$^+$ cells can be localized in the subplate (H) and in the lower cortical layers (I), while still present, in a small fractional proportion, in the meninges and meningeal substructure (G). Inset in I show a single-channel image of migrating CherryRed$^+$ cells. Scale bars: 20 μm.

It is important to note that CherryRed$^+$ cells were never detected in the upper cortical layers at 15 and 24-48 hours, indicating that they did not migrate directly from the outer meninges into the cerebral cortex, but rather that the transduced meningeal cells migrated via the VZ into the brain parenchyma (Figure 3.8, 3.9).

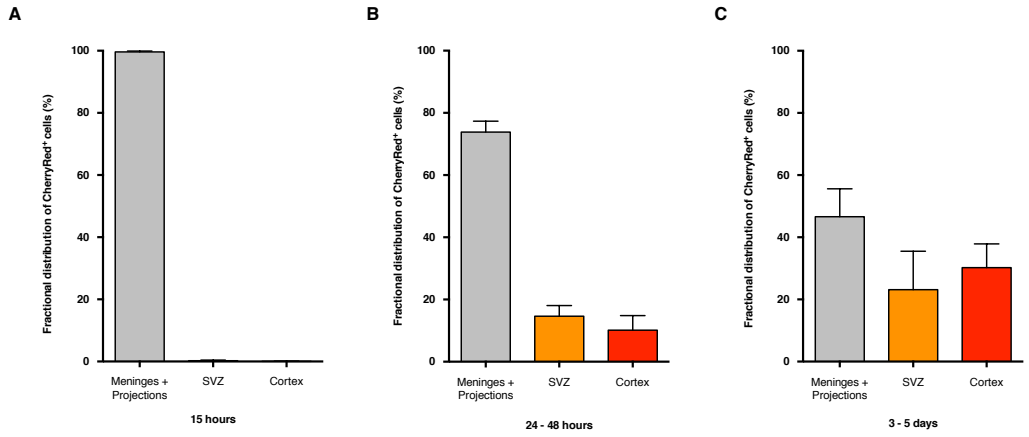

Figure 3.9. Quantification of the CherryRed$^+$ signal distribution in the time course analysis. Distribution of CherryRed$^+$ signal between the meninges and meninges substructures (gray bar), ventricular and subventricular zone (orange bar) and cortex (red bar) at 15 hours (A, n = 4), 24-48 hours (B, fractional distribution (%): meninges 73.8 ± 3.5, SVZ 14.6 ± 3.4, cortex 10.2 ± 4.7; n = 4) and 3-5 days (C, fractional distribution (%): meninges 46.6 ± 9.0, SVZ 23.2 ± 12.3, cortex 30.2 ± 7.7; n = 3) after meningeal transduction.

At later timepoints, *i.e.* 21-30 days post-transduction, most of the CherryRed$^+$ cells were detectable in the upper cortical layers II-IV (Figure 3.10A-C) and were mostly confined to the retrosplenial and visual motor cortex regions (Figure 3.10D,E).

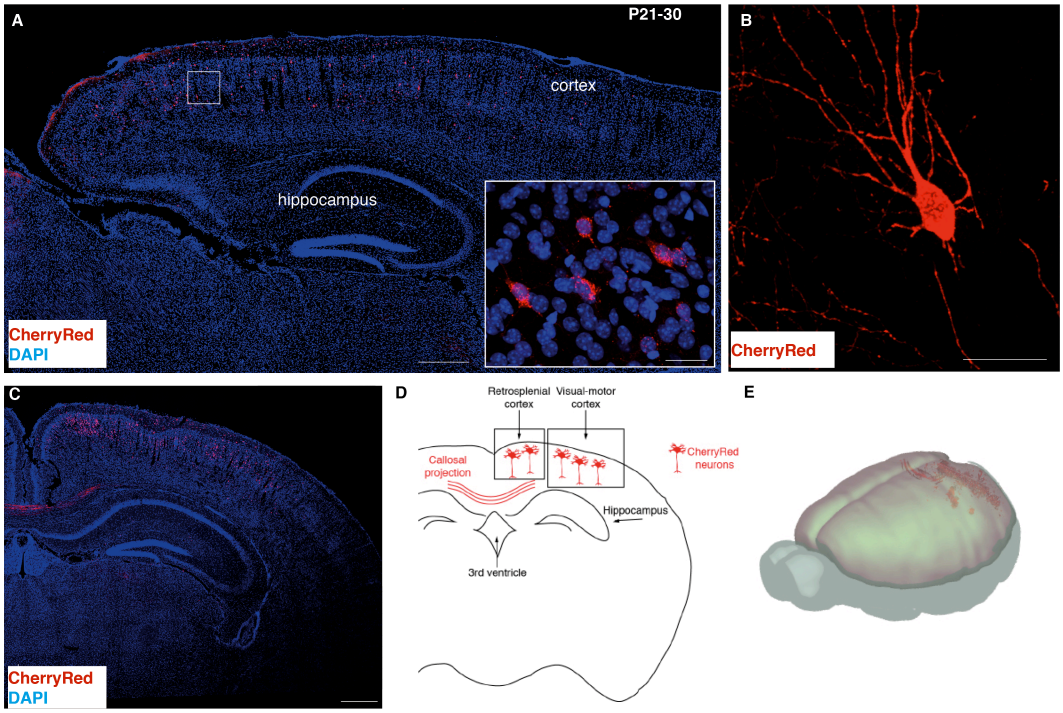

Figure 3.10. Brain sections of a P21-30 CD1 mouse injected in the meninges with LV-CherryRed at P0.

A, Sagittal and section of P21-30 CD1 mice injected with the LV-CherryRed in the meninges at P0, showing the distribution of CherryRed$^+$ cells in the upper cortical layers at 21-30 days after meningeal transduction. Inset in A represents higher magnification of the boxed area. **B,** CherryRed$^+$ neuron detected in the upper layer of the cortex, 21-30 days after meningeal transduction. **C,D,** Coronal section (C) and schematic representation (D) of P21-30 CD1 mice injected with the LV-CherryRed in the meninges at P0, showing the distribution of CherryRed$^+$ cells in the upper cortical layers at 21-30 days after meningeal transduction. **E,** 3D reconstruction of the brain distribution of CherryRed$^+$ cells at 21-30 days after meningeal injection of the LV-CherryRed in P0 CD1 mice, depicting the localization of CherryRed$^+$ cells in the retrosplenial and visual motor cortices. Scale bars: 500 μm (A, C), 50 μm (inset in A; B).

3.2.2 – Control experiments on viral transduction

We wanted to verify the specificity of the LV injection approach, *i.e.* the absence of non-specifical labelling due to LV diffusion in other (non meningeal) regions of the brain.

To this aim, we first performed PCR analysis to detect LV-mRNA. PCR analysis on meningeal, cortical and ventricular samples collected 15 hours after meningeal injection of a LV-plvx-GFP at P0. The data revealed that the plvx-GFP-lentiviral

mRNA was detectable in the meninges and not in the brain parenchyma of the cortex or VZ (Figure 3.11).

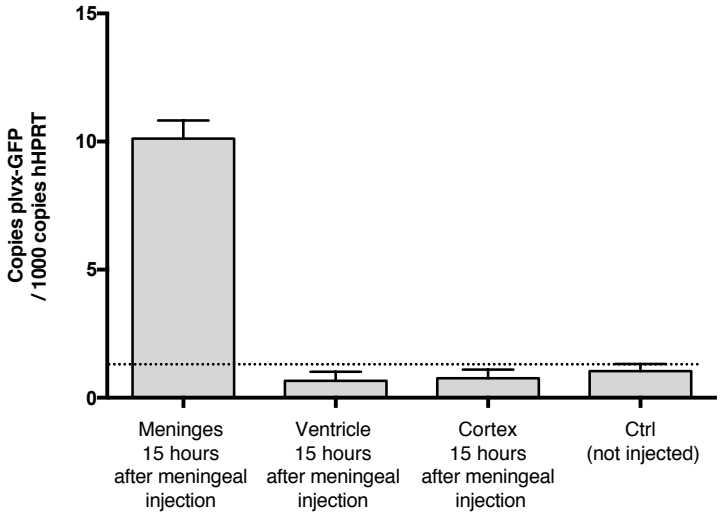

Figure 3.11. RNA expression analysis of plvx-GFP copies in P0 CD1 mice injected in the meninges with the LV-plvx-GFP.

15 hours post meningeal injection, the expression of plvx-GFP is detectable only in the meninges and not in the VZ or cerebral cortex (meninges 10.1 ± 0.5, cortex 0.8 ± 0.2, ventricle 0.7 ± 0.3, Ctrl (not injected) 1.0 ± 0.2; n= 6 pups).

To test whether low amounts of LV-particles might have diffused into the cerebrospinal fluid (CSF) to the lateral ventricles or other cortical regions, we sampled CSF of P0 pups at 12 hours after the injection of the LV-Cherry into the meninges or intrathecally (as a positive control). Using a highly sensitive ELISA, we readily detected the LV-protein p24 (a non-glycosylated capsid protein commonly use to titer the LV preparations, Geraerts et al., 2006) in the CSF after intrathecal injection; on the contrary, no p24 was detectable after injection in the meninges (Figure 3.12). These results confirm that the LV-particles do not diffuse into the CSF after meningeal transduction.

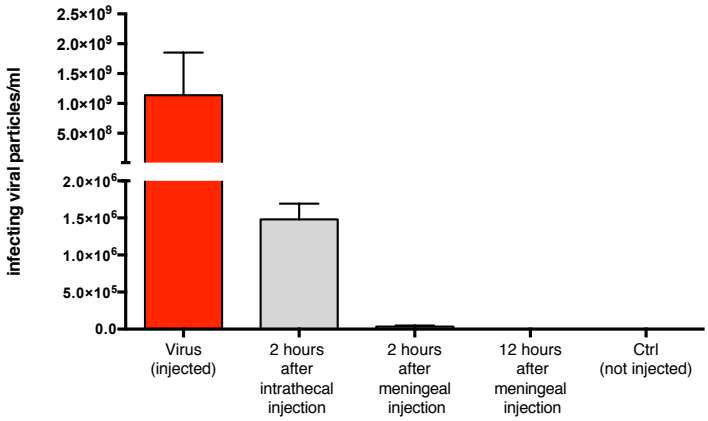

Figure 3.12. Concentration of LV-particles in the CSF of P0 CD1 pups injected with LV-CherryRed into the cisterna magna (intrathecal injection) or meninges.

At 12 hours after injection of the LV-CherryRed in meninges, no LV particles were detectable in the CSF (virus injected $1.1x10^9 \pm 3.6x10^8$, 2 hours after intrathecal injection $1.5x10^6 \pm 0.2x10^6$, 2 hours after intra-meningeal injection $0.3x10^5 \pm 0.1x10^5$, 12 hours after meningeal injection 0; n= 7 pups).

Finally, to exclude that our LV-transduction technique target somehow the ventricle and/or the VZ during the injection, we compared the distribution of CherryRed$^+$ cells after injection of LV-CherryRed into the meninges *versus* LV-plvx-GFP directly into the ventricle. By this approach we could distinguish whether the patterns of expression depended on the site of injection. Injection of LV-plvx-GFP into the lateral ventricle resulted in a completely different distribution pattern than the one observed after CherryRed$^+$ meningeal transduction. Indeed, already 15 hours after injection into the lateral ventricle, GFP$^+$ cells were detected in the VZ and SVZ throughout the forebrain and, notably, in the epithelium of the choroid plexus (Figure 3.13A). As expected, some days later, we observed labelled cells in the RMS and granular layer of the OB, a distribution that was never seen following meningeal injection (Figure 3.13B). This experiment demonstrated that meningeal injection resulted in a completely different pattern of expression compared to the direct injection in the ventricle, thus confirming the specificity of our labelling technique.

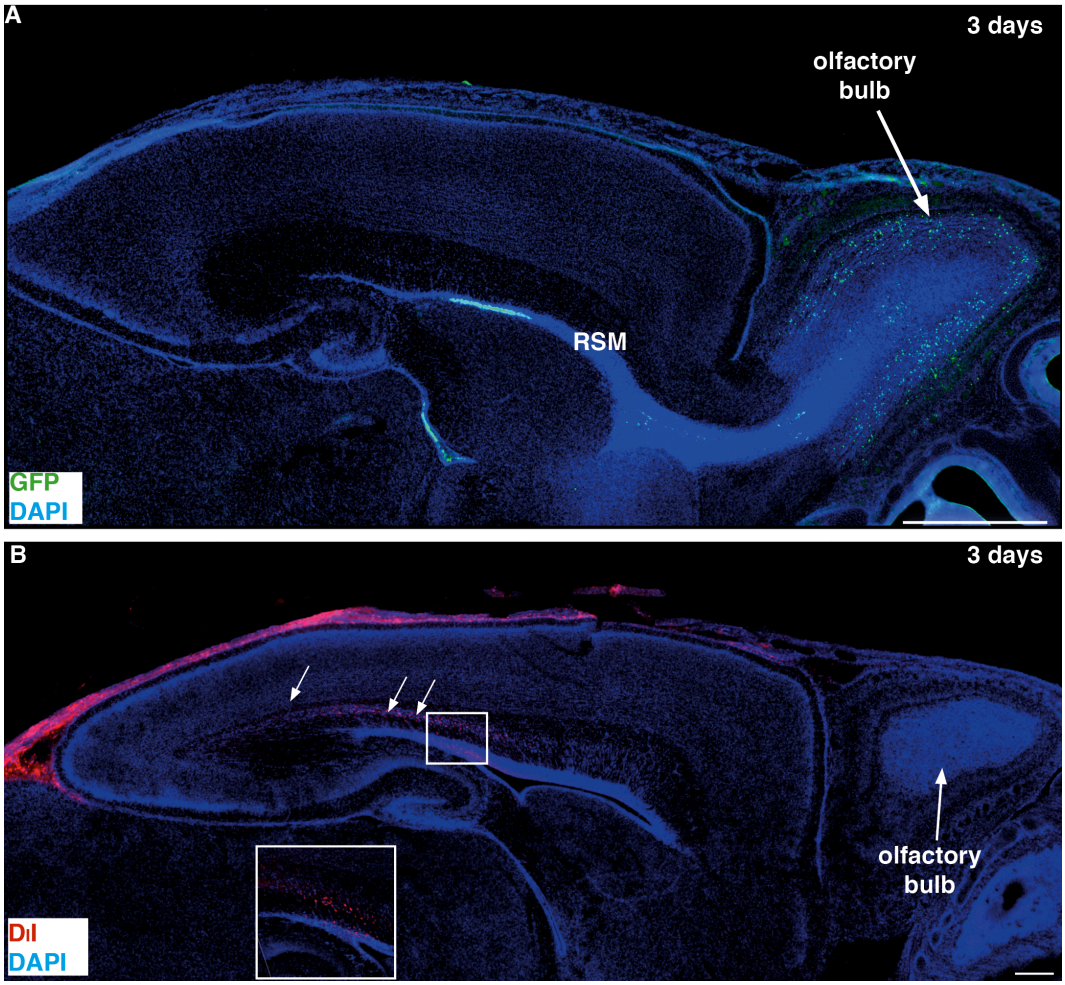

Figure 3.13. Brain section of P3 CD1 mice injected in the ventricle with LV-plvx-GFP, compared to the injection of DiI in the meninges.

3 days after ventricle injection, the plvx-GFP$^+$ cells are found in the rostral migratory stream (RSM) and granular layer of the olfactory bulb (A, arrow). These regions were never labelled with the injection in the meninges (B). Arrows in B show the DiI$^+$ cells in the VZ. Inset in B represent a higher magnification of the boxed area. Scale bar: 500 μm.

Overall, our tests on the quality of our labelling method demonstrated that i) we have been able to devise a technique that label exclusively meningeal cells; ii) the LV-CherryRed injection was the most appropriate technique to trace meningeal cells in time; iii) LV-CherryRed transduction was specific to the meningeal layer and iv) the LV did not diffuse in the CSF or in the other tissues after the injection.

3.2.3 – Time course analysis: meningeal labelling by RFP plasmid electroporation

To confirm the results obtained with LV-CherryRed transduction, we performed time course analysis employing RFP electroporation to label meningeal cells. This analysis revealed a distribution of RFP$^+$ cells similar to that obtained after meningeal injection of LV-CherryRed, with cells residing in the meninges at early time points, then migrating along the meningeal substructures (Figure 3.14B), the fornix and the choroid plexus (Figure 3.14C,D), reaching the VZ and the lower layers of the cortex (Figure 3.14E).

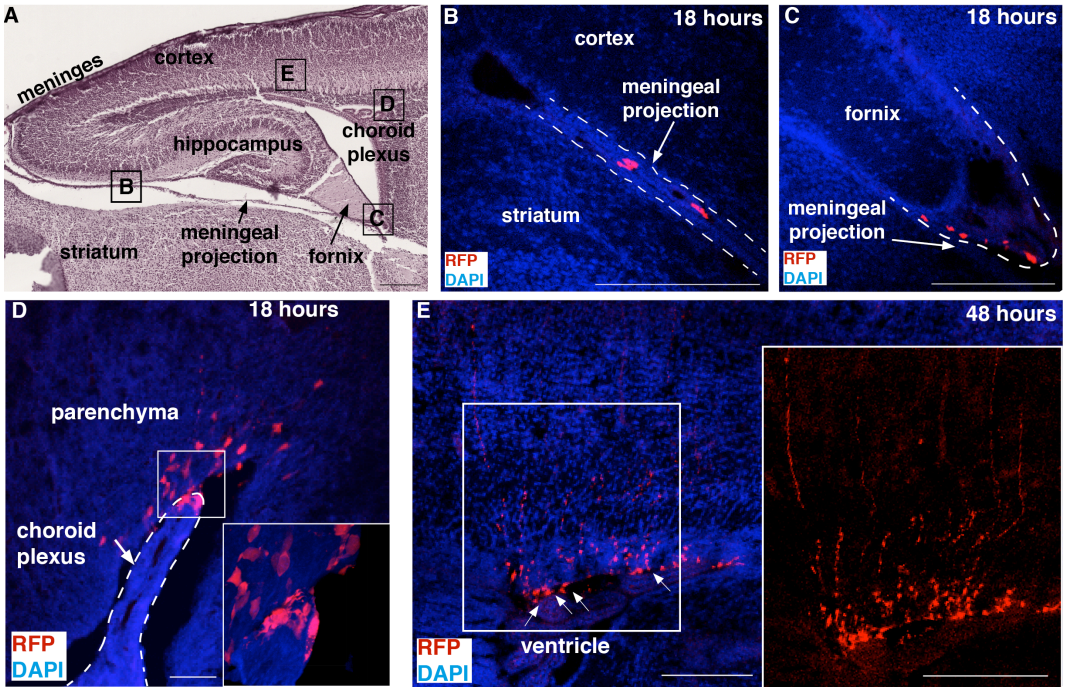

Figure 3.14. Electroporation of the meninges with an RFP encoding plasmid P0 C57/BL6 mouse.

A, H&E staining of a sagittal section through a P0 C57/Bl6 mouse brain, indicating the brain location of RFP$^+$ labelled cells. **B-D,** Path of migration of RFP$^+$ cells through the meningeal substructures entering the brain (B), around the fornix (C) and the choroid plexus (D), at 18 hours after electroporation. Inset in D represents a higher magnification of the boxed area, showing RFP$^+$ cells wrapping the choroid plexus. **E,** RFP$^+$ cells migrated to the VZ and SVZ by 48 hours after electroporation. Arrows in A-D indicate the specific regions; arrows in E point to RFP$^+$ cells lining the ventricle. The inset in E shows the red color channel image of a larger magnification of the boxed area. The dashed white lines in B-D delineate the meningeal substructure (B,C), and choroid plexus (D). Scale bars: 200 µm (A,E), 20 µm (B-D).

3.2.4 – Time course analysis: meningeal labelling by DiI injection

To further confirm the migratory path observed with both LV-CherryRed transduction and RFP electroporation, we injected the DiI in the meninges of P0 pups, and checked the distribution of DiI-labelled cells in the brain cortex at several time points. After 1, 6 and 12 hours, we observed that the DiI^+ cells were confined to the meninges only, and were not detected in the brain parenchyma (cortex) or VZ (3.15A). At 24-48 hours and 7 days, we detected DiI^+ cells in the VZ and cortex (Figure 3.15B,C respectively), confirming the results obtained with the other meningeal labelling techniques (LV transduction, Figure 3.7; plasmid electroporation, Figure 3.13).

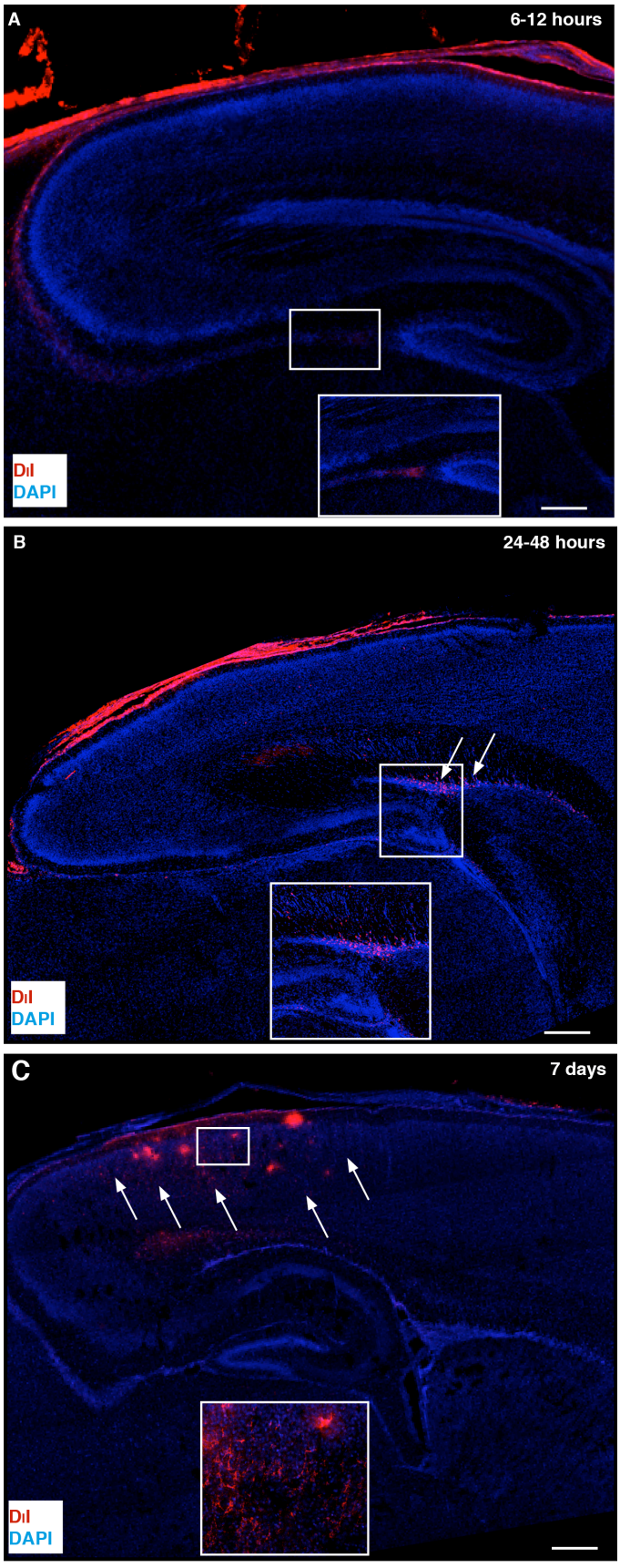

Figure 3.15. Time course analysis of DiI labelled meningeal cells.

Analysis at 6-12 (A) and 24-48 hours (B), and 7 days (C) after DiI injection in the meninges confirmed the migration path described with meningeal LV transduction and electroporation. **A,** At 6-12 hours after meningeal injection, DiI^+ cells were confined to the meninges and meningeal substructures and undetectable in the cortical parenchyma. **B, C,** By 24-48 hours after meningeal injection, DiI^+ cells migrated to the SVZ (B) and preplate, reaching the cortex by 7 days (C). Scale bars: 200 μm (A-C).

3.2.5 – Meningeal cells migrate by locomotion from the ventricle to the cortex: time lapse imaging analysis

To observe more directly the cell migratory behavior from the meninges to the meningeal substructures and lateral ventricle, we performed time-lapse movies of fresh cortical slice preparations. We therefore injected the DiI in the meninges of P0 pups and analysed them at 24 and 72 hours after the injection (see Material and methods, 2.8). This analysis revealed that DiI^+ cells migrated from the ventricle to the subplate and thereafter to the lower and upper cortical layers. We observed that DiI^+ cells migrated via locomotion in a discontinuous, saltatory manner with an average speed of 51.58 ± 12.98 μm/hour (n = 60 cells ; Figure 3.16).

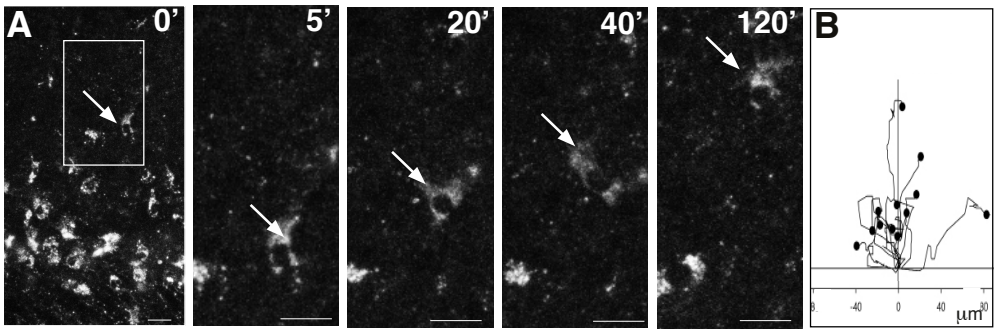

Figure 3.16. Time-lapse movies of fresh cortical slice preparations.

A, Time-lapse movies of cortical slices at 24 hours after meningeal injection of the fluorescent dye DiI in P0 CD1 mice. Arrows show DiI^+ cells movement within the timeframes. The brain area shown in the time lapse micrographs are higher magnification of the boxed area in A. **B,** Graph represents analysis of cell-motility tracks by time-lapse imaging of DiI^+ cells. Cell tracks were determined by cell nuclei position, migration origin was superimposed at the zero-cross point. Scale bars: 20 μm.

Our experiments demonstrate that meningeal cells, labelled by means of different tracers with a newly developed technique, are able to migrate from their location, outside the brain parenchyma, to the upper cortical layers. Migrating meningeal cells follow their path along the meningeal substructures, underneath the hyppocampus,

along the choroid plexus, to the VZ and the subplate. The migration occurrs by locomotion, in a saltatory manner, from the VZ to the cortical layers. These data represent the first report of postnatal migration of meningeal cells to the cortex, thus highlighting the relevance of these findings.

3.3 – Embryonic origin of meningeal migrating cells

We next investigated at which embryonic age the cells that migrated postnatally from the meninges to the cortex were generated. Using LV-CherryRed, we transduced the meninges of P0 pups, from CD1 mothers that had received a single injection of EdU either at E12.5, E13.5, E14.5, E16.5, E17.5 or E18.5, or pups that had received a single EdU injection at P0. We analysed the fraction of cells that had incorporated EdU at P21-30 (Figure 3.17B).

Our analysis indicated that most of the CherryRed$^+$ cells in the cortex incorporated EdU between E13.5 and E16.5, while less than 1% of them were labelled beyond E17.5 (Figure 3.17A,C). These findings clearly suggest that the large majority of the meningeal migrating cells are embryonically derived. Notably, these CherryRed$^+$ cells showed long-term retention of embryonically administrated EdU, suggesting that they remain largely quiescent during the postnatal period.

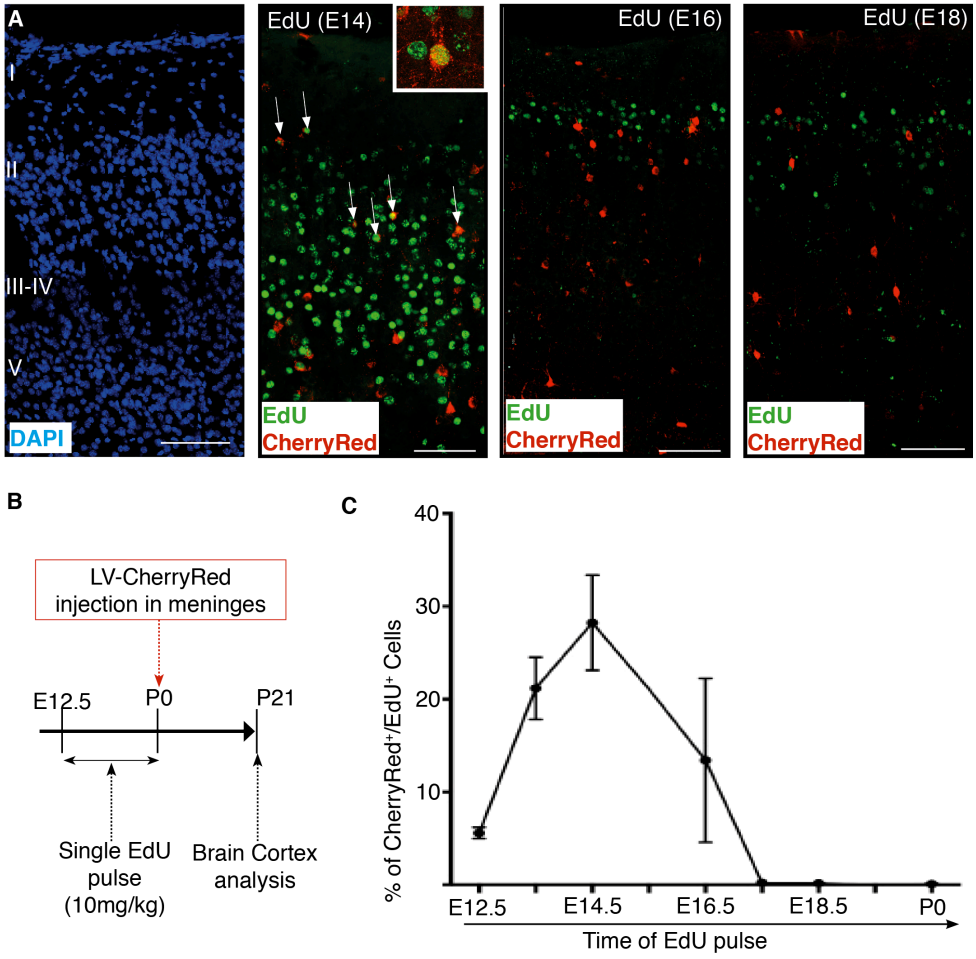

Figure 3.17. Birthdating experiments.

A, Brain sections of P21-30 CD1 mice that received a single intraperitoneal injection of EdU at embryonic day (E) E14.5, 16.5 or 18.5, respectively, and meningeal injection of the lentiviral vector expressing CherryRed at P0. Staining for EdU (green) and CherryRed$^+$ cells (red) in the cortex, showing that meningeal-derived CherryRed$^+$ cells (arrows) migrating in the cortex, preferentially incorporated EdU at E14. Left panel: representative section showing nuclei (DAPI staining, blue) to visualize the cortical layers. Inset in the E14 panel represents higher magnification of CherryRed$^+$ / EdU$^+$ cells. **B,** Schematic representation of the birthdating experiment. A single dose of EdU was administered to pregnant CD1 mothers either at E12.5, E13.5, E14.5, E16.5, E17.5, or E18.5, or in pups at P0. After birth, meninges of these P0 CD1 pups were transduced using LV-CherryRed. Analysis was performed at P21. **C,** Quantification of CherryRed$^+$/ EdU$^+$ cells in brain section of P21-30 CD1 mice pulsed with EdU at different embryonic days and injected in the meninges with the LV-CherryRed at P0, showing that most of the CherryRed$^+$ cells incorporated EdU between E13.5 and E16.5 (n = 12). Scale bars: 200 μm.

3.4 – Meningeal cells differentiate into functional cortical neurons

3.4.1 – Phenotype characterization

We observed cells derived from the meninges migrating from their location, outside the brain parenchyma, along the meningeal substructures, to the VZ in 24-48 hours, to the lower cortical layer in 3-5 days and finally reaching the upper cortical layer by P21-30.

We studied the phenotype of the migrating CherryRed^{+} meningeal cells at these time points. At 24-48 hours after transduction with LV-CherryRed in the meninges of P0 mice, we observed that most CherryRed^{+} cells in the VZ do not expressed the radial glia markers Blbp and GLAST (Figure 3.18A,B). By day 3-5, CherryRed^{+} cells in the cortex expressed the neuronal precursor markers doublecortin (Francis et al., 1999) and HuC/D (Wakamatsu and Weston, 1997) (Figure 3.18C,D).

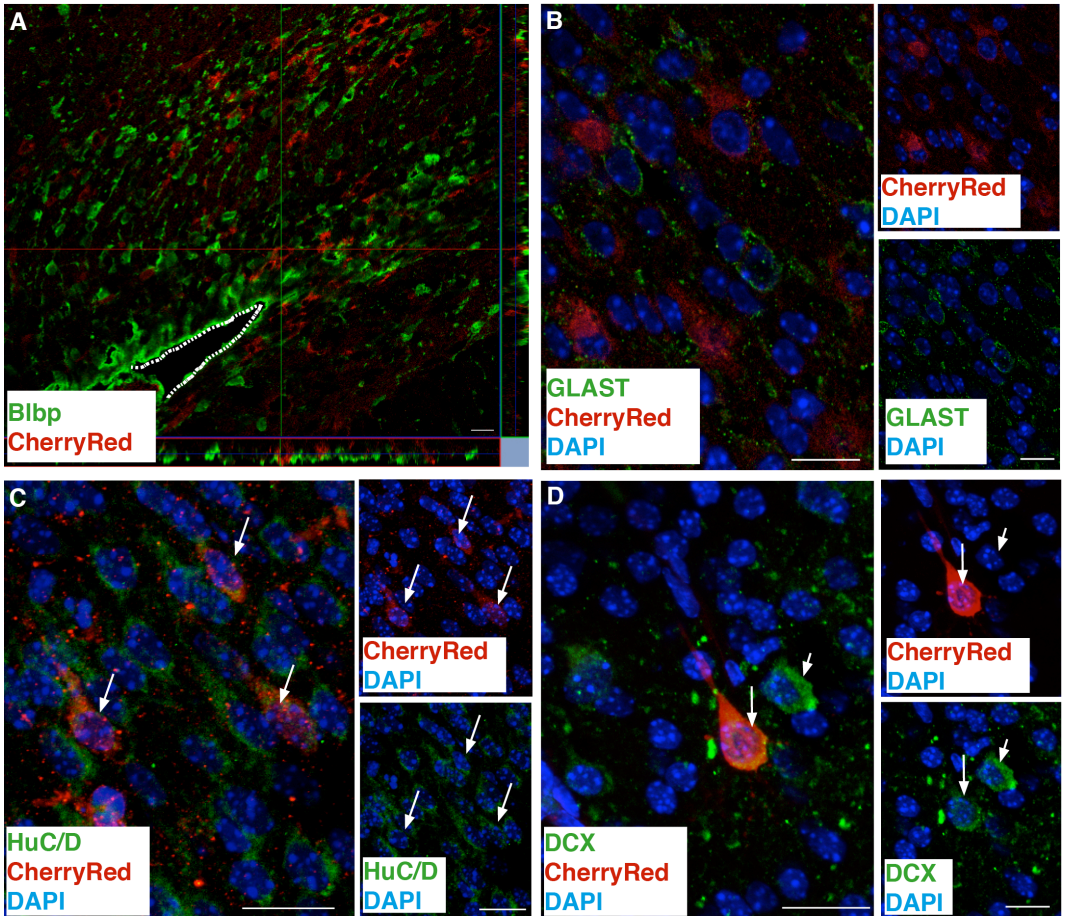

Figure 3.18. Meningeal-derived migrating cells express neuronal precursors marker.

A, Orthogonal projection of Z-stack confocal image of CherryRed^{+} cells (red) in the SVZ, at 24 hours after meningeal lentiviral injection and stained for the radial glia marker Blbp (green), showing that

CherryRed+ cells do not co-express Blbp. The dashed white line delineates the ventricle. **B,** Brain sections of CD1 mice at 48 hours after CherryRed LV transduction of the meninges, showing that CherryRed+ cells (red) did not express the radial glia marker GLAST. **C,D,** Brain sections of CD1 mice at 3 days after CherryRed lentiviral transduction of the meninges, showing that CherryRed+ cells (red) express the marker of immature and post-mitotic neurons HuC/D (C, arrows) and doublecortin (DCX) (D, arrows). Scale bars: 20 μm.

We also characterized and quantified the phenotype of the meningeal-derived CherryRed$^+$ cells at P21-30, when neuronal pruning had already occurred (Bandeira et al., 2009; Table 1). Up to 75% of the parenchymal CherryRed$^+$ cells in the cortex showed a neuronal morphology and expressed the pan-neuronal marker NeuN (Figure 3.19A,G). CherryRed$^+$ cells did not express the astrocyte marker GFAP, the oligodendrocyte precursor marker NG2, or the microglia marker Iba1 (Figure 3.19 D-F,G). Among the NeuN$^+$ cells, 75.8% expressed Satb2, a marker of neurons that establish callosal projections (Alcamo et al., 2008; Figure 3.19B,G), while 20.5% expressed the interneuron marker GAD65/67 (Figure 3.19C,G). Thus, meningeal cells migrated into the brain parenchyma and generated primarily cortical neurons.

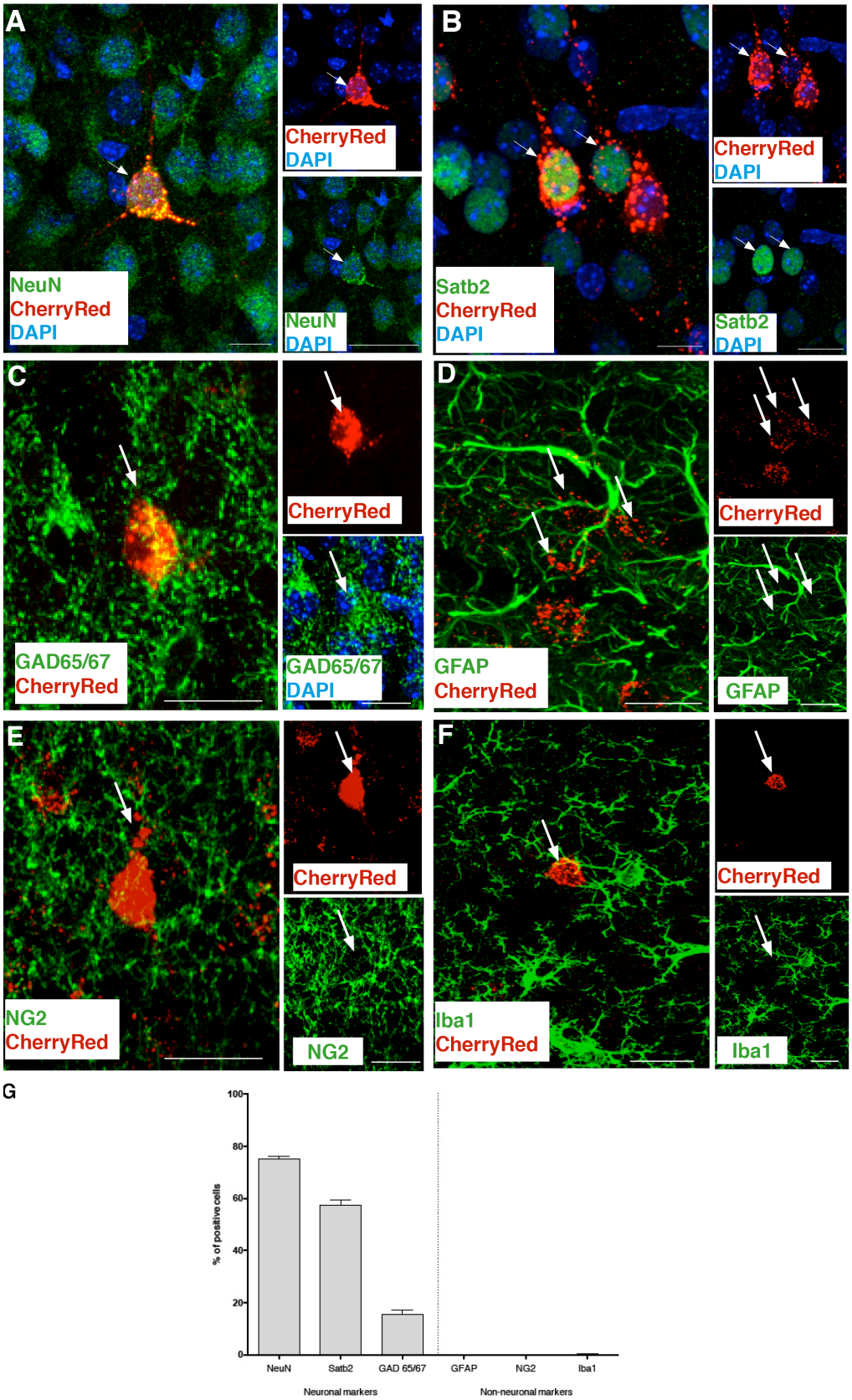

Figure 3.19. Quantification of markers expression in P21-30 CD1 mice injected with LV-CherryRed at P0.

A-F, Brain section of a P21-30 CD1 mouse injected in the meninges with LV-CherryRed at P0, showing that CherryRed+ cells in the cortex co-express the pan-neuronal marker NeuN (A, arrow). Among those, they express the neuronal marker Satb2 (B, arrow) and GAD65/67 (C). The markers GFAP (D), NG2 (E), and Iba1 (F) were minimally or not expressed. **G,** Quantification of markers expression in P21-30 CD1 mice, injected in the meninges with the LV-CherryRed at P0. The dashed line divides the neuronal (NeuN, Satb2, GAD65/67) from the non-neuronal (GFAP, NG2, Iba1) markers. Scale bars: 20 μm.

Similar results were obtained when we studied the fate of the migrating meningeal cells by electroporating a Cre-expressing plasmid in the meninges of neonatal Rosa26-lox-stop-lox-YFP mice (Srinivas et al., 2001). Cre expression by transfected meningeal cells results in constitutive YFP expression, allowing tissue-specific fate mapping of meningeal cells. At 21-30 days after Cre electroporation, YFP^+ cells were detected in the upper cortical layers I-IV Up to 77.3% of the parenchymal YFP^+ cells in the cortex showed a neuronal morphology and expressed the pan-neuronal marker NeuN (Figure 3.20A,G), while a small percentage (12.1%) of YFP^+ cells in the subpial region expressed the astrocyte marker GFAP (Figure 3.20D,G). These $GFAP^+$ cells were detected only by using the anti-GFAP polyclonal antibody (rabbit anti-GFAP, DAKO), while they could not be detected using the monoclonal antibody (mouse anti-GFAP, Sigma). Among the $NeuN^+$ cells, 69.1% expressed Satb2 (Figure 3.20B,G), while the remaining 13.2% expressed the interneuron marker GAD65/67 (Figure 3.20C,G). YFP^+ cells did not express the oligodendrocyte precursor marker NG2 or the microglia marker Iba1 (Figure 3.20E-G).

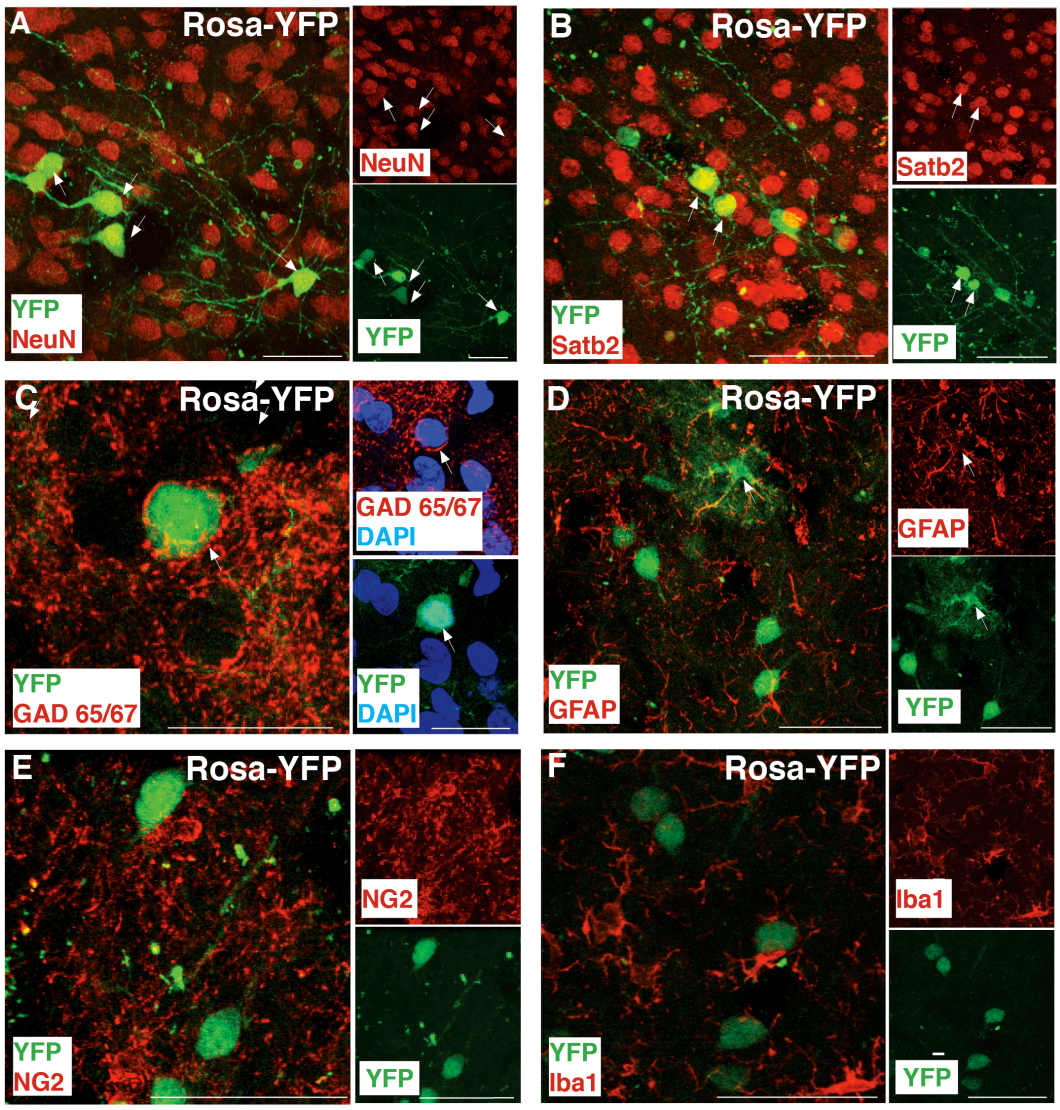

Figure 3.20. Quantification of markers expression in P21-30 Rosa-YFP mice electroporated with the Cre plasmid at P0.

A-F, Brain section of a P21-30 Rosa-YFP mouse electroporated in the meninges with Cre plasmid at P0, showing that YFP$^+$ cells in the cortex co-express the pan-neuronal marker NeuN (A, arrows). Among those, they express the neuronal marker Satb2 (B, arrows) and GAD65/67 (C, arrow). A minor proportion expressed the marker GFAP (D). The markers NG2 (E) and Iba1 (F) were minimally or not expressed. Scale bars: 20 μm.

3.4.2 – Electrophysiological analysis

In collaboration with Prof. Michele Giugliano and Dr. Gabriella Panuccio, Theoretical Neurobiology & Neuroengineering Lab, University of Antwerp (Belgium)

We then assessed whether the meningeal-derived neurons in the cortex are functional in terms of electrogenic cellular properties. We carried out

electrophysiological experiments in acute cortical slices, obtained from P15-30 CD1 mice injected at P0 in the meninges with LV-CherryRed (Figure 3.21A). By using video microscopy and whole-cell patch-clamp, we targeted CherryRed$^+$ cells and found that meningeal-derived neurons acquired an excitable electrical phenotype, similar to that of endogenous cortical neurons (Figure 3.21B,C), as they robustly discharged trains of action potentials (APs) upon intracellular current injection (Figure 3.21C). Studied under current-clamp, the AP shape and the frequency of fired APs during a sustained discharge of CherryRed$^+$ cells were largely reminiscent of fast-spiking neurons rather than regularly firing neurons (Markram et al., 2004). Indeed, compared to the control group of randomly selected neighboring cortical neurons, the half-width duration of individual APs and their trajectory steepness during the AP falling phase were significantly different in CherryRed$^+$ cells (Figure 3.21D,E). These features support a rapid repolarization of the cell membrane potential after each AP and are therefore consistent with the cell's ability to sustain faster AP discharge rates, as we observed (control: peak 27 ± 2.9 Hz (range: 13.5 - 42 Hz), n=9; CherryRed$^+$ cells: peak 54.8 ± 7 Hz (range: 26.6 - 85.2 Hz), n=9; p=0.001). These features are also consistent with a larger slope of the input-output response curves, obtained in CherryRed$^+$ cells upon quantifying the AP discharge frequency in response to injected external currents with increasing amplitude (control: 0.12 ± 0.02 Hz/pA; LV-Cherry+: 0.27 ± 0.04 Hz/pA; p=0.004) (Fig. 2W). These input-output properties indicate a higher sensitivity to small changes in the input and thus a higher gain for transmission of information, compared to control. Notably, these electrophysiological features are reminiscent of new neurons generated by adult NSCs, which are hyper-excitable when compared to mature neurons (Ge et al., 2008; Marín-Burgin et al., 2012; Mongiat et al., 2009).

Importantly, CherryRed$^+$ cells were functionally integrated in the cortical local synaptic microcircuitry. Indeed, patch-clamp recordings revealed spontaneous and evoked postsynaptic potentials (Figure 3.21F), elicited by extracellular electrical stimulation of connected neighbouring cells. In addition, the pharmacological dissection of synaptic responses by selective antagonists of glutamatergic and GABAergic receptors and across varying holding potentials, demonstrated integration of CherryRed$^+$ cells in both excitatory and inhibitory local microcircuits (Figure 3.21G). Overall, these findings demonstrate that meningeal-derived cells that

migrated into the cortex: (i) acquired an intrinsic excitable electrical phenotype of cortical neurons *in vivo*, without prior *in vitro* manipulation by experimental conditions or reprogramming (Espuny-Camacho et al., 2013; Karow et al., 2012a); and (ii) became functionally integrated in established cortical networks as they received functional synaptic inputs from both excitatory and inhibitory neurons. The biological significance of postnatal meningeal derived neurons remains unknown. As hypothesized for NSC-derived adult-born neurons, we speculate that they may contribute to information processing differently from mature neurons in the same circuit, and may promote plasticity of the existing circuitry by making new synaptic contacts with mature neurons (Ming and Song, 2011).

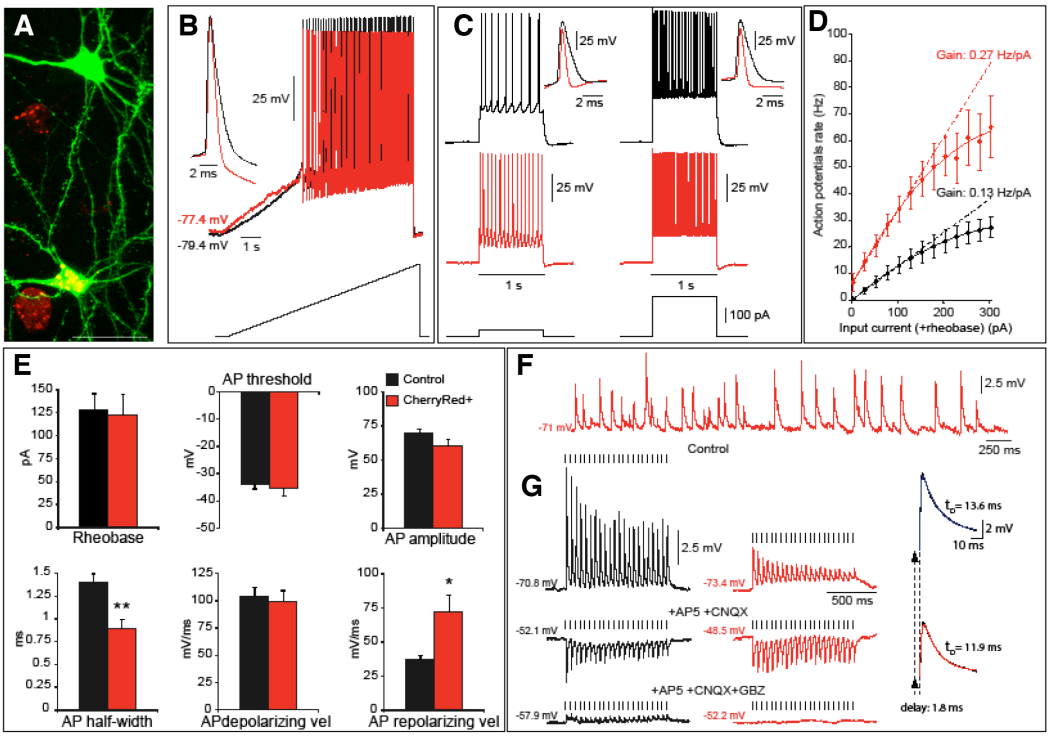

Figure 3.21. Electrophysiological (somatic, whole-cell patch-clamp) recordings in acute cortical tissue slices, obtained from P15-21 CD1 mice injected at P0 in the meninges with LV-CherryRed.

A, Confocal microscopy image showing a control patched (biocytin$^+$) cortical neuron (green, top) and a neighboring biocytin$^+$/CherryRed$^+$ (yellow) double positive cell. **B,** CherryRed$^+$ cells can generate action potentials (APs). Superimposed responses of a control cortical (black trace) and a CherryRed$^+$ cell (red trace) to intracellular injection of a 10 s-long depolarizing current ramp (30 pA/s). The two cell types exhibit comparable rheobase currents, as indicated by the similar time of onset of the first APs generated upon depolarization. On the left are the first APs generated by two cell types, superimposed at a faster time-scale. **C,** CherryRed$^+$ cells exhibit a firing behavior similar to cortical neurons. Firing properties of a cortical neuron (black trace) and a CherryRed$^+$ cell (red trace) in

response to intracellular injection of 1 s-long depolarizing current steps of 50 pA (left) and 300 pA (right). On the right of each traces the first APs generated upon intracellular injections of the current steps are shown, superimposed at a faster time-scale. Note the shorter duration and faster repolarizing velocity of the AP generated by the CherryRed$^+$ cell. **D,** CherryRed$^+$ cells display a higher sensitivity to external current than cortical neurons. Average frequency-current curves obtained from cortical neurons (black trace) and CherryRed$^+$ cells (red trace) showing the higher average firing frequency (peak 54.8 ± 7 Hz, n = 9) and slope (*i.e.* gain) (0.27 ± 0.04 Hz/pA, n = 9) of the latter. **E,** Histogram plots summarizing the active membrane properties of control neurons and of CherryRed$^+$ cells. CherryRed$^+$ cells (red bars) generate APs that are significantly faster than those generated by control cortical neurons (black bars) (AP half-width 0.9 ± 0.1 ms, n = 9, ** $p< 0.005$; repolarizing velocity 72.4 ± 12.4 mV/ms, n = 9,* $p< 0.01$). **F,** Spontaneous post-synaptic potentials (PSPs) recorded in current-clamp from a CherryRed$^+$ cell at resting membrane potential (indicated). **G,** Evoked PSPs (ePSPs) recorded in response to 1 s-long trains (20 Hz) of extracellular electrical square pulses (indicated by the vertical solid lines above each trace). Cortical neurons (black trace) and CherryRed$^+$ cells (red trace) respond similarly to electrical stimulation under different pharmacological paradigms. During perfusion with standard ACSF (Control), the ePSPs are depolarizing at resting potentials (~ -70mV). Subsequent co-application of ionotropic glutamatergic receptor blockers (+AP5 +CNQX) abolishes the depolarizing ePSPs and unmasks the presence of hyperpolarizing responses. The evoked hyperpolarizing potentials are abolished by the GABAAR antagonist gabazine (GBZ). Values on the left of each trace indicate the holding membrane potential (Vm), which was increased by intracellular current injection during the pharmacological isolation of the inhibitory responses, to alter and reverse their ionic driving force. In fact, an amplitude reversal obtained for synaptic currents at holding potentials around the Nernst potential for Chloride, in the range of [-70 ; -50] mV, demonstrates the activation of GABAAR. Note the depolarized Vm during pharmacological isolation of the inhibitory responses. The evoked hyperpolarizing potentials are abolished by the GABAAR antagonist gabazine (GBZ). Curve on the right shows the first PSPs evoked in control condition at a faster time-scale, to emphasize the similar temporal latency of the responses generated by the two cell types (right vertical dash line) from the stimulus onset (arrowhead and left vertical dashed line), as well as the similar (de)activation kinetics, quantified by fitting the responses to a double exponential function (blue: Cortical neuron; red: CherryRed$^+$ cell). The decaying time constant tD is indicated at the right of each evoked PSP.

3.5 – Lineage tracing of meningeal migrating cells

3.5.1 - Meningeal migrating cells are mostly different from RGCs

We observed meningeal-derived functional neurons in the cortex of adult mice, and we thus explored whether these neurons were derived from the radial glial (RG) lineage.

We generate a transgenic line for RGCs lineage tracing by intercrossing GLAST-CreERT2 mice (expressing a tamoxifen-inducible Cre in RGCs, (Mori et al., 2006) with the Rosa26-lox-stop-lox-YFP reporter line (Srinivas et al., 2001), yielding GLAST-YFP mice, in which RGCs and their descendants are permanently labelled upon tamoxifen injection (Mori et al., 2006). Injection of pregnant GLAST-YFP mice with tamoxifen at E13.5 resulted in the expected labelling of cortical neurons and ventricular RGCs in newborn pups (Figure 3.22A). We also detected GLAST-derived YFP^+ cells located perivascularly in the meningeal substructures, *i.e.* in 7.90 ± 0.87% of the meningeal cells (Figure 3.22B).

Then, to investigate whether meningeal cells were derived from the RG-GLAST lineage, we injected LV-CherryRed in the meninges of P0 Glast-YFP pups treated with tamoxifen at E13.5. Analysis of the brains at P21-30 that only ~13% of the $CherryRed^+$ cells, which had migrated from the meninges into the cortex, were YFP^+ (Figure 3.22C-E, Figure 3.24). Hence, only a minor proportion of the meningeal-derived cells were derived from the $GLAST^{+}$ cells generated at E13.5.

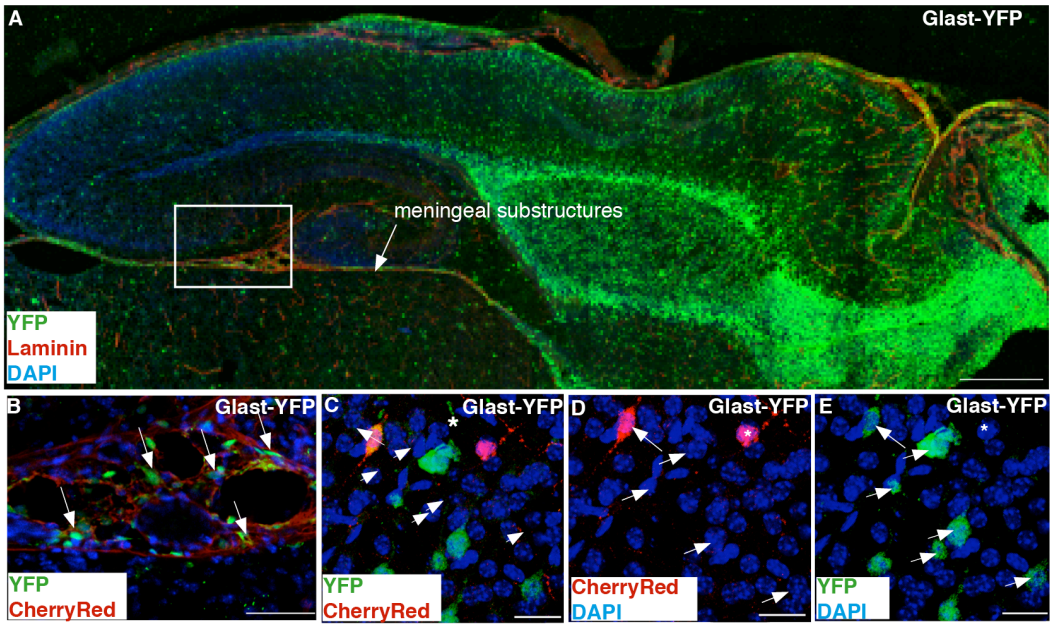

Figure 3.22. Brain section of GLAST-YFP mice induced with tamoxifen at E13.5.

A,B, Sagittal brain section of a P0 GLAST-YFP mouse treated with tamoxifen at E13.5, showing the distribution of RG-derived YFP$^+$ (green) cells (A). Laminin staining (red) reveals consistent accumulation of GLAST-derived YFP$^+$ cells located in the perivascular space of the meningeal substructures (B). B represents higher magnification of the boxed area in A; arrows in B indicate the perivascular YFP$^+$ cells. **C-E,** Brain section of a P21-30 GLAST-YFP mouse treated with tamoxifen at E13.5 and injected in the meninges with LV-CherryRed at P0. Most of the GLAST-derived YFP$^+$ (C,E; green, arrowheads) cells do not co-express CherryRed$^+$ (C,D; red, asterisk). A minor proportion of migrated cells (arrows) were YFP$^+$/CherryRed$^+$ double positive. Scale bars: 200 µm (A), 20 µm (B-E).

To further investigate this issue, since Nestin is expressed in neural precursors including RGCs during embryonic development (Lendahl et al., 1990), we assessed whether the meningeal-derived cortical neuronal population was generated from Nestin$^+$ neurogenic cells in the meninges. We therefore intercrossed Nestin-CreERT2 mice (Lagace et al., 2007) with the Rosa26-lox-stop-lox-YFP reporter line (Srinivas et al., 2001, yielding Nestin-YFP mice). Induction of Cre expression with tamoxifen at E13.5 resulted in labelling of cortical cells of P21-30 mice (Figure 3.23A).

We transduced the meninges of P0 pups of the Nestin-YFP with the LV-CherryRed after induction with tamoxifen at E13.5, and analysed the mice at P21-30. This analysis revealed that only 4.5% of the CherryRed$^+$ cells were also labelled by YFP (Figure 3.23C,D; Figure 3.24). This is consistent with the low proportion of YFP$^+$ cells in the meningeal substructures of P0 Nestin-YFP pups after treatment with tamoxifen at E13.5 (~1% of the meningeal cells, Figure 3.23B).

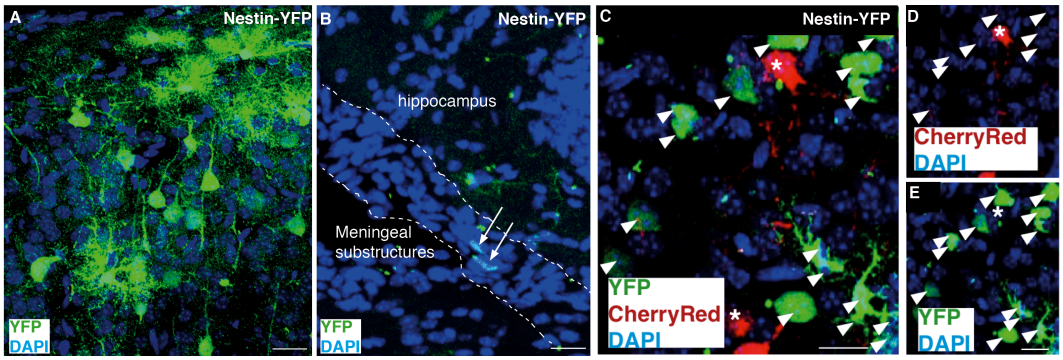

Figure 3.23. Sagittal brain sections of Nestin-YFP mice induced with tamoxifen at E13.5.

A,B, Brain section of P21-30 Nestin-YFP mice treated with tamoxifen at E13.5. Pictures show the distribution of Nestin-derived YFP$^+$ (green) cells in the cortex (A) and meningeal substructures (dashed line in B). Arrows in B indicate YFP$^+$ cells in meningeal substructures. Dashed line in B delineate meningeal substructures. **C-E,** Brain section of a P30 Nestin-YFP mouse treated with tamoxifen at E13.5 and injected in the subarachnoidal space with LV-CherryRed at P0, showing that most of the Nestin-derived YFP$^+$ cells (green, arrowheads) cells do not co-express CherryRed$^+$ (red, asterisk). Scale bars: 20 μm.

Thus, a large fraction of cortical cells derived from meninges was a separate cell population, distinct from GLAST$^+$ and Nestin$^+$ lineages generated at E13.5 (Figure 3.24). Since we pre-labelled cortical cells in these experiments, these results argue against cell fusion as a possible mechanism.

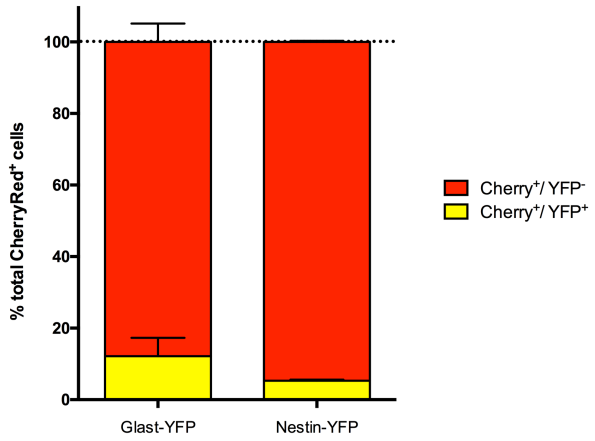

Figure 3.24. Quantification of the proportion of CherryRed$^+$/ YFP$^+$ cells in Glast-YFP and Nestin-YFP mice injected with LV-CherryRed at P0.

Bars represent the % of the CherryRed$^+$/YFP$^+$ among the total CherryRed$^+$ (yellow bars, 12.2 ± 2.9; n=3 and 5.3 ± 0.3; n=3 in the Glast-YFP and Nestin-YFP mouse lines respectively) cells in the cortex of P21-30 GLAST-YFP (right bar) or Nestin-YFP (left bar) mice. Mice were treated with tamoxifen at E13.5.

3.5.2 – Neurogenic meningeal cells belongs to PDGFRß$^+$ lineage

3.5.2.1 – Unperturbed conditions: PDGFRß –YFP

Meninges are a highly vascularized tissue, containing blood vessels that are in close contact with perivascular cells expressing PDGFRß (Figure 3.25A-C) (Lindahl et al., 1997). Considering that PDGFRß-expressing meningeal cells have been suggested to have neurogenic potential *in vitro* (Nakagomi et al., 2011, 2015b), we explored whether the neurogenic cells that migrated from the meninges and

differentiated to neurons in the cortex were derived from the PDGFRß-expressing lineage, using a gene fate mapping strategy.

We therefore intercrossed PDGFRß-Cre mice (Foo et al., 2006) with the Rosa26-lox-stop-lox-YFP reporter line (Srinivas et al., 2001, yielding PDGFRß-YFP mice). We detected YFP expression in perivascular cells of the meninges (Figure 3.25D,E). Moreover, in line with reports that PDGFRß is also expressed by a subset of neuronal progenitors in the VZ (Ishii et al., 2008a; Williams et al., 1997), we detected YFP expression in the VZ and in cortical cells of P0 pups (Figure 3.25D,F).

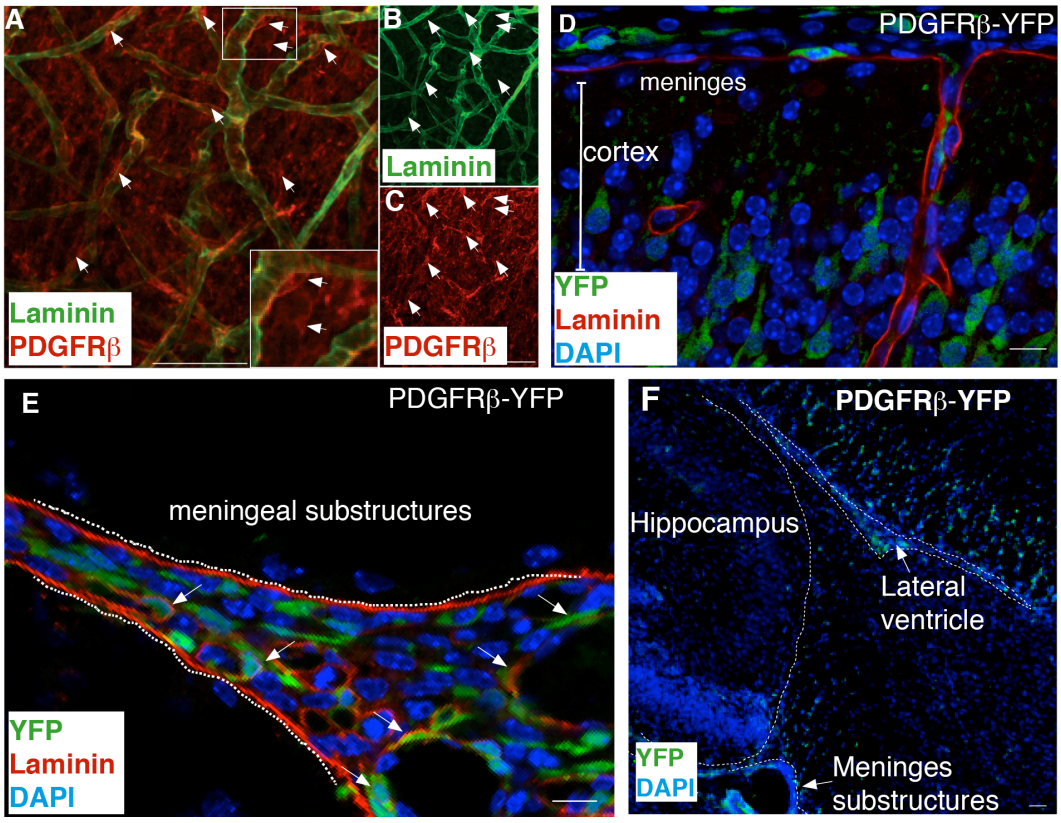

Figure 3.25. PDGFRß expression in the perivascular cells.

A-C, Whole-mount staining of meninges (top-view) of a P0 CD1 mouse. Arrows indicate the PDGFRß cells (red, panel C) lining the vessels (laminin, in green, panel B) in meninges. **D,E,** Brain cortex (D) and meningeal substructure (E) of a P0 PDGFRß-YFP mouse stained for YFP (green) and the vascular basement membrane marker laminin (red) showing that YFP^+ cells are localized in the brain parenchyma (A) and in the meningeal substructure close to the vessels as indicated by the arrows. Cell nuclei are visualized by DAPI staining (blue). **F,** Brain section of a P0 PDGFRß-YFP mouse stained for YFP showing the distribution of YFP^+ cells alongside the lateral ventricle (dashed line). Cell nuclei are visualized by DAPI staining. Scale bars: 50 µm (A-E), 20 µm (F).

In P21-30 PDGFRß-YFP mice, we observed that up to 93.4% of the cortical YFP^+ cells expressed the pan-neuronal marker NeuN and, of those, 87.6% expressed Satb2 (Figure 3.26A,B,G; Table1). The results obtained using the unperturbed PDGFRß-YFP mouse model showed that a fraction of the cortical neurons was derived from the $PDGFRß^+$ lineage. Moreover, the distribution of PDGFRß-YFP^+ cells in the meninges and meningeal substructures of the brain suggested a possible meningeal origin of the PDGFRß-YFP^+ cortical neurons. However, since other cell types in the brain also expressed PDGFRß, this experiment did not exclude the possibility that PDGFRß-YFP^+ cortical neurons originated from other PDGFRß-YFP^+ cells in non-meningeal brain regions.

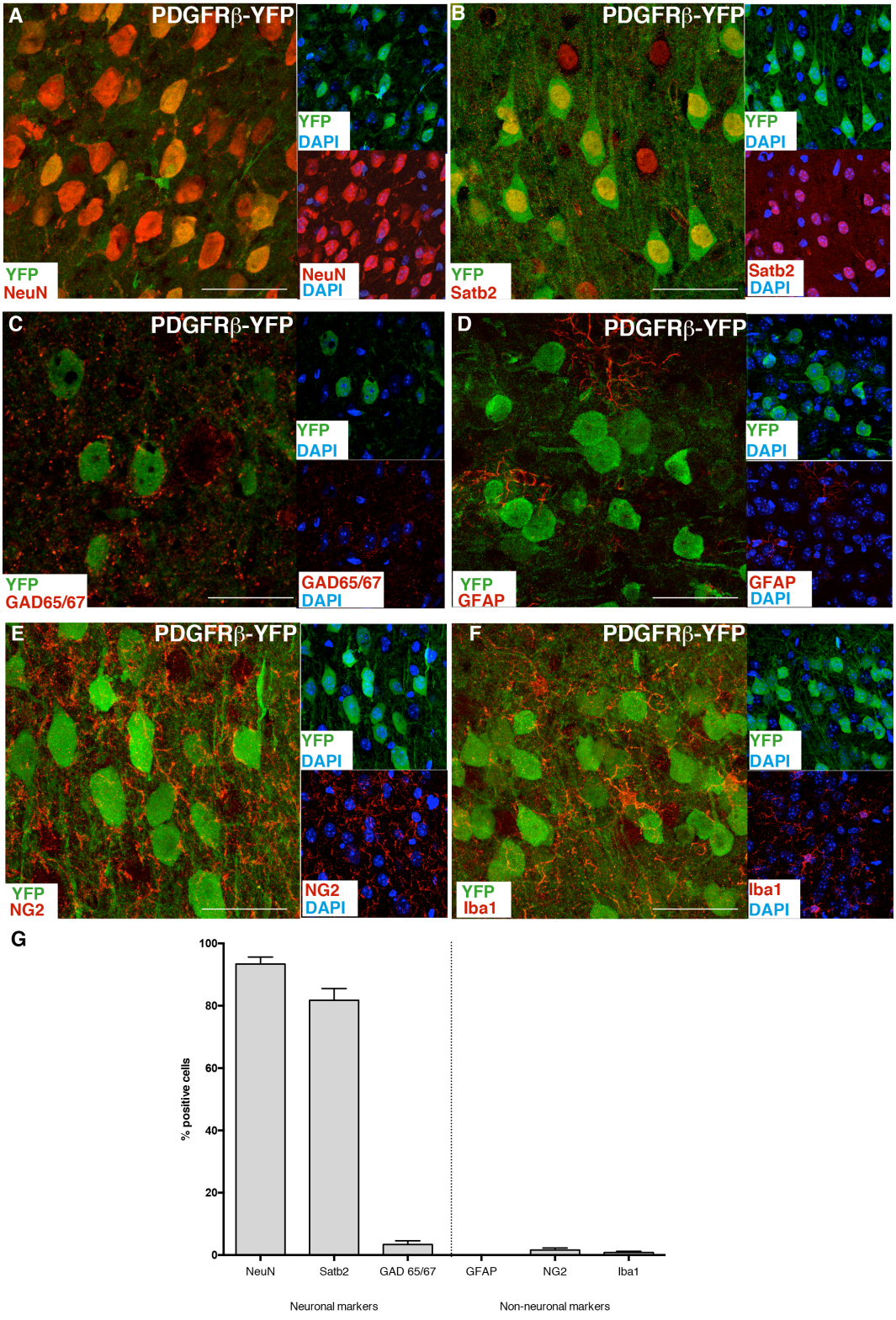

Figure 3.26. Quantification of markers expression in P21-30 PDGFRß –YFP transgenic mice.

A-F, Brain section of a P21-30 PDGFRß-YFP mouse, showing that YFP^{+} cells in the cortex co-express the pan-neuronal marker NeuN (A). Among those, they express the neuronal marker Satb2 (B) and GAD65/67 (C). A minor proportion expressed the marker NG2 (E). The markers GFAP (D)

and Iba1 (F) were minimally or not expressed. **G,** Quantification of markers expression in P21-30 PDGFRß-YFP mice. The dashed line divides the neuronal (NeuN, Satb2, GAD65/67) from the non-neuronal (GFAP, NG2, Iba1) markers. Scale bars: 50 µm.

3.5.2.2 – Tissue specific gene-fate mapping: PDGFRß-Cre injected with LV-Brainbow 1.0(L)

To explore whether PDGFRß-YFP^{+} cortical neurons originated from meningeal-derived cells, we devised a technique to selectively label only the PDGFRß -expressing cells in the meninges: a tissue-specific gene fate mapping. We therefore injected a LV expressing the meninges of newborn PDGFRß-Cre mice with a lentiviral vector expressing Brainbow 1.0(L) (Figure 3.27A). This Brainbow 1.0(L) reporter is engineered such that it expresses the red fluorescent protein tdTomato before, and the blue and yellow fluorescent proteins CFP or YFP, respectively, after Cre-mediated excision of the floxed stop cassette (Figure 3.27A). Once switched on, the persistent expression of CFP/YFP allows fate mapping of the PDGFRß-expressing meningeal cells and their progeny.

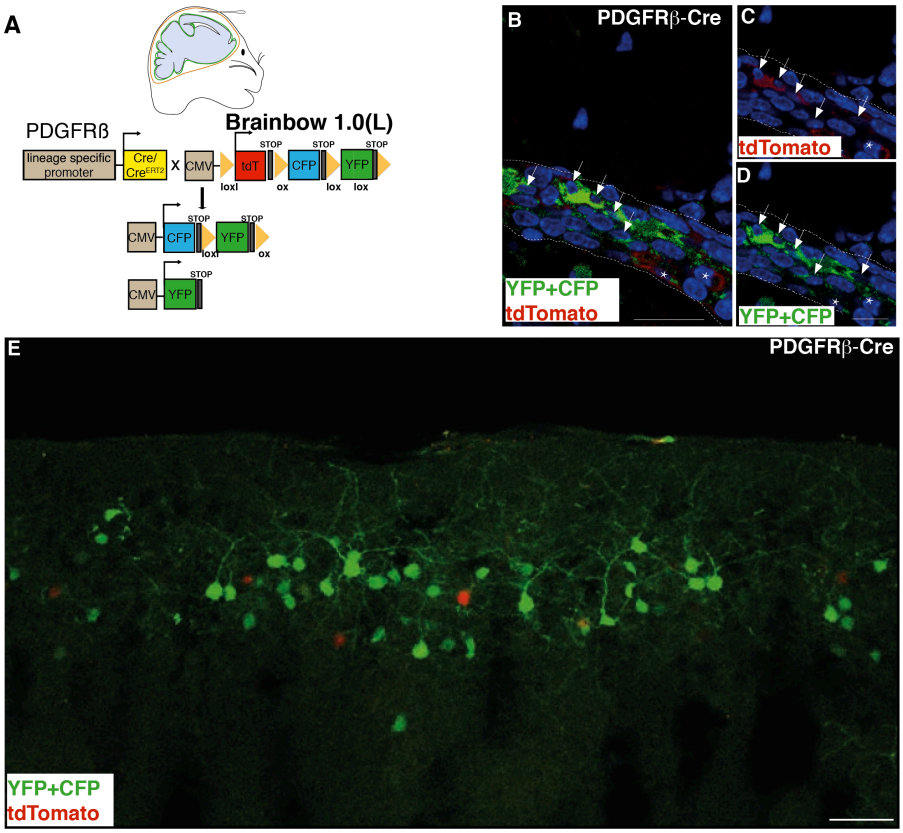

Figure 3.27. Recombination in PDGFRß-Cre injected with the LV-Brainbow 1.0(L) at P0.

A, Schematic representation of the Cre induced recombination of LV-Brainbow 1.0(L) injected in the meninges of lineage-specific PDGFRß -Cre expressing mice. The red/brown and green line in the brain scheme denotes the outer layer (arachnoid) and inner layer (pia mater) of the meninges, respectively. The different colors after Cre-recombination are shown. CFP, cerulean fluorescent protein; tdT, tdTomato fluorescent protein; YFP, yellow fluorescent protein; CMV, cytomegalovirus promoter. **B-D,** Brain section of a P1 PDGFRß-Cre mouse, injected at P0 with a LV- Brainbow 1.0(L) in the meninges, stained for YFP/CFP (green, B,D; arrows) and tdTomato (red, B,C; asterisks), showing that Cre-mediated excision of the lox-stop-lox sites of the Brainbow 1.0(L) (green) vector occurs already after 24 hours at the level of the meninges substructure (dashed line). **E,** Brain cortex of a P21-30 PDGFRß-Cre mouse, injected at P0 with LV-Brainbow 1.0(L) in the meninges, stained for YFP/CFP and tdTomato, showing that the meningeal cells that migrated into the cortical layer I-IV were mostly PDGFRß-Cre derived YFP^+/CFP^+ (green) cells. Scale bar: 100 μm.

Since tdTomato-labelled cells represent cells in which Cre was not active, we focused on the cells expressing CFP/YFP. For unknown reasons, we observed background recombination, leading to the formation of 19.6% CFP^+/YFP^+ cortical cells in wild type (WT) mice, not expressing Cre (Table 1). A similar background activation of the LV-Brainbow 1.0(L) was observed when transducing this reporter in cultured cells (not shown).

At 20-24 hours after lentiviral transduction with the LV-Brainbow 1.0(L) in the meninges of PDGFß-Cre P0 pups, we found that 79.9 ± 9.7% of all labelled cells in the meninges expressed CFP/YFP (Figure 3.27B-D). A similar proportion of recombined cells (76.7% of all labelled cells) was detected at 21-30 days after LV-Brainbow 1.0(L) transduction. CFP^+/YFP^+ cell were located in the upper cortical layers I to IV (Figure 3.27E), of which 82.2% expressed NeuN (Figure 3.28A,B,H; Table 1). $GFAP^+$, $NG2^+$ and $Iba1^+$ cells were minimally or not detected amongst the CFP^+/YFP^+ cortical cells (Figure 3.28E-H; Table 1). Most of the CFP^+/YFP^+ neurons expressed Satb2 (86.0%) (Figure 3.28A,C,H), while a small proportion were $GAD65/67^+$ interneurons (13.4%) (Figure 3.28D,H). Thus, perivascular $PDGFRß^+$ cells migrate from their meningeal location into the brain parenchyma and differentiate primarily to $Satb2^+$ neurons in the upper layer I-IV of the cortex. In the PDGFRß-Cre line transduced with Brainbow 1.0(L), ~25-30% of the labelled cortical cells were $tdTomato^+$, indicating that Cre was not active in these cells at the time of the transduction. This may be due to insufficient Cre activity in a fraction of

PDGFRß$^{+}$ cells and/or may suggest that they did not uniformly express, at least at the time of transduction, PDGFRß.

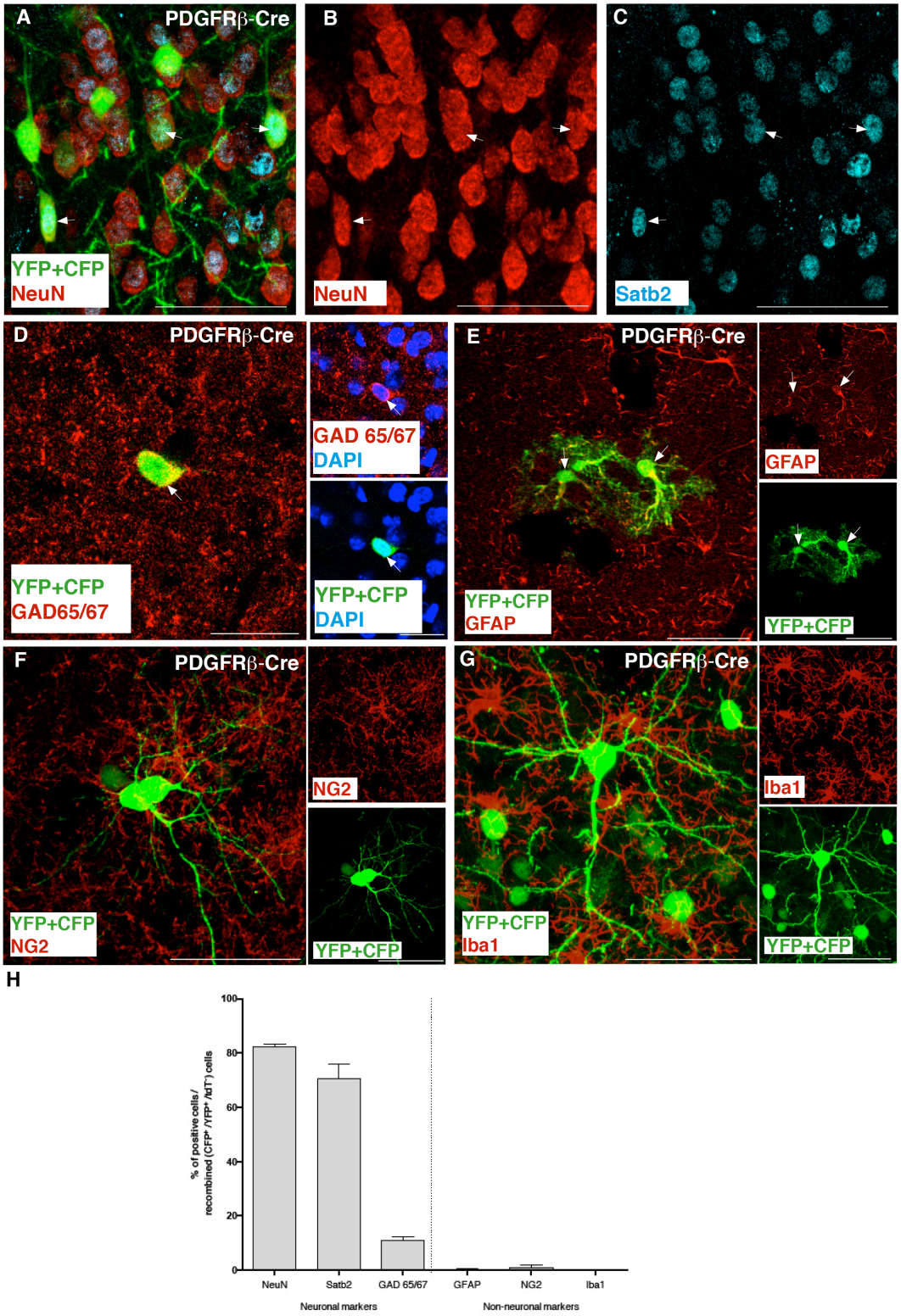

Figure 3.28. Quantification of markers expression in P21-30 PDGFRß-Cre transgenic mice injected with LV-Brainbow 1.0(L) at P0.

A-G, Brain section of a P21-30 PDGFRß-Cre mouse injected with LV-Brainbow 1.0(L) in the meninges, showing that YFP^+ cells in the cortex co-express the pan-neuronal marker NeuN (A,B, arrows). Among those, they express the neuronal marker Satb2 (A,C, arrows) and GAD65/67 (D, arrow). A minor proportion expressed the marker GFAP (E, arrow). The markers NG2 (F, arrow) and Iba1 (G) were minimally or not expressed. **H,** Quantification of markers expression in P21-30 PDGFRß-Cre mice injected with LV-Brainbow 1.0(L) in the meninges at P0. The dashed line divides the neuronal (NeuN, Satb2, GAD65/67) from the non-neuronal (GFAP, NG2, Iba1) markers. Scale bars: 50 μm.

3.6 – Developmental origin of meningeal-derived neurons

3.6.1 – Unperturbed conditions: Wnt1-YFP

To identify the developmental origin of meningeal-derived neurons we employed a gene fate mapping strategy. Forebrain meninges have been described to be originated from the neural crest (Johnston, 1966; Couly et al., 1992, 1995; Siegenthaler and Pleasure, 2011a). Previous report showed efficient labelling of forebrain meninges and meningeal brain substructures by using a Wnt1-Cre reporter line (Jiang et al., 2002). We therefore selected the Wnt1-Cre-driven mouse lines, which expressed the Cre recombinase at least in meningeal cells (in addition to other cell types), to investigated whether meningeal cells and meningeal derived neurons could be developmentally originated from neural crest.

In order to genetically visualize the Wnt1 population, we intercrossed the constitutive Wnt1-Cre driver line with the Rosa26-lox-stop-lox-YFP reporter line (Srinivas et al., 2001, yielding Wnt1-YFP mice), resulting in permanent labelling of Wnt1 expressing cells and their descendants. In agreement with previous observations (Jiang et al., 2002), we detected YFP expression in E16 embryos and P0 pups in the diencephalon, hippocampus, forebrain meninges, meningeal brain projections, and lining the choroid plexus (Figure 3.29A,B). Furthermore, we also observed YFP^+ cells, arranged in columns of radially oriented cells, spanning from the ventricle to the upper cortical layers (Figure 3.29B). In adult Wnt1-YFP mice, we observed a similar distribution of YFP^+ cells, however the columns of YFP^+ cells in the cortex were no longer detectable in the ventricular and subventricular zone, but became restricted to the upper cortical I to IV layers (Figure 3.29C). Notably we identified, at all the analysed stages, YFP^+ cells in the cortical layers (insets in Figure 3.29).

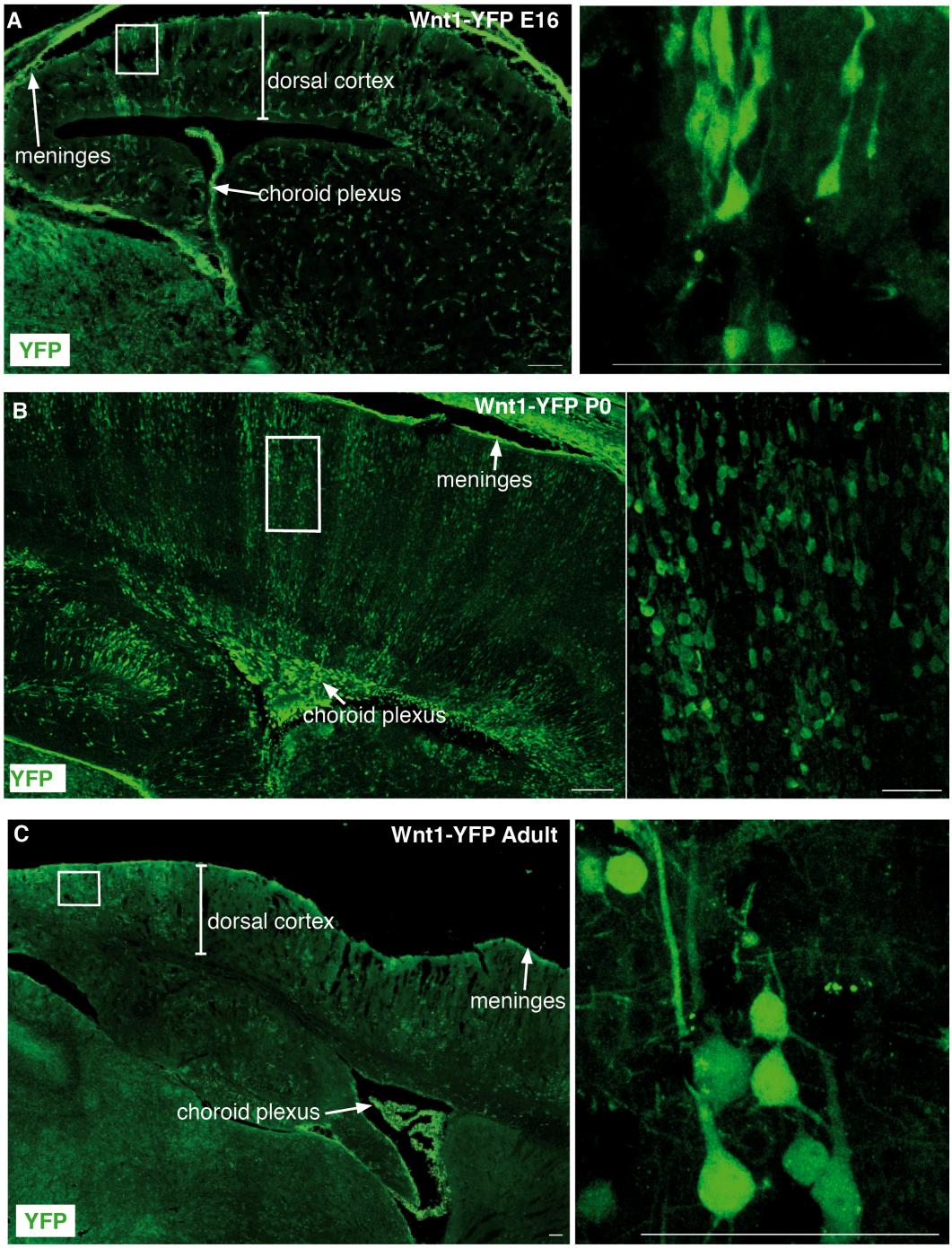

Figure 3.29. YFP expression in the Wnt1-YFP transgenic line.

Reconstructions of sagittal sections of E16 (A), P0 (B) and adult (C) Wnt1-YFP brains. Insets are an higher magnification of the boxed areas, showing the presence of YFP^+ cells in the cortex at each developmental stages. Scale bars: 100 μm

When analysing the phenotype of the Wnt1-derived YFP^+ cells in the cortex of the P0 pups, we observed that ~84% of the YFP^+ cortical cells expressed the marker

HuC/D, while ~77% expressed the marker DCX, meaning that the majority of the Wnt1$^+$ cells in the cortex are immature neurons (Figure 3.30).

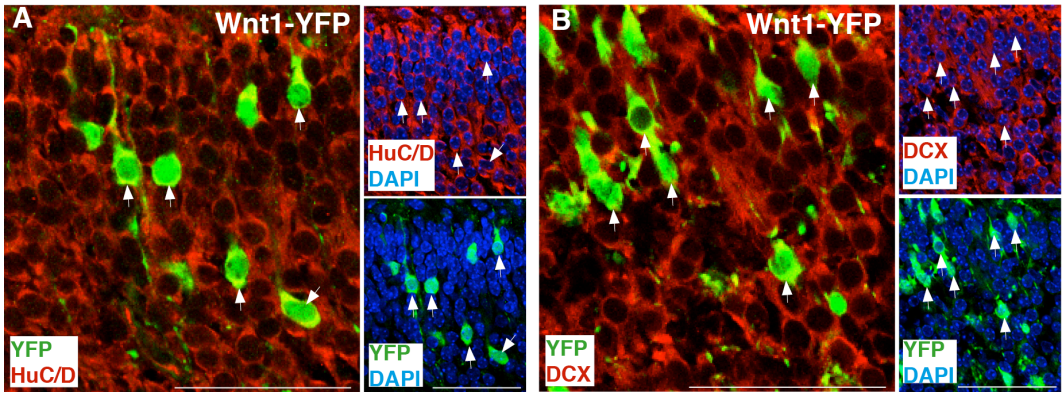

Figure 3.30. Neuronal progenitor expression in Wnt1-YFP P0 mice.

YFP$^+$ cells in Wnt1-YFP P0 mice are present in the cortex and expressed neuronal progenitor markers HuC/D (arrows in A) and DCX (arrows in B). Scale bars: 50 µm

In P21-30 adult Wnt1-YFP mice, we found that up to 82.7% of the cortical Wnt1-YFP$^+$ cells expressed the pan-neuronal marker NeuN and, of those, 60.8% expressed Satb2 (Figure 3.31A-C,H), while ~12% expressed GAD65/67 (Figure 3.31D,H). Rarely, the expression of the markers GFAP, NG2 and Iba1 was detected (Figure 3.31E-H).

The results obtained using unperturbed Wnt1-YFP mouse model (*i.e.* non injected with a LV or electroporated) showed that a fraction of the Wnt1-YFP$^+$ cells generated cortical neurons. However, since other cell types in the brain also expressed Wnt1, this experiment did not exclude the possibility that Wnt1-YFP$^+$ cortical neurons originated from other YFP$^+$ cells in non-meningeal brain regions.

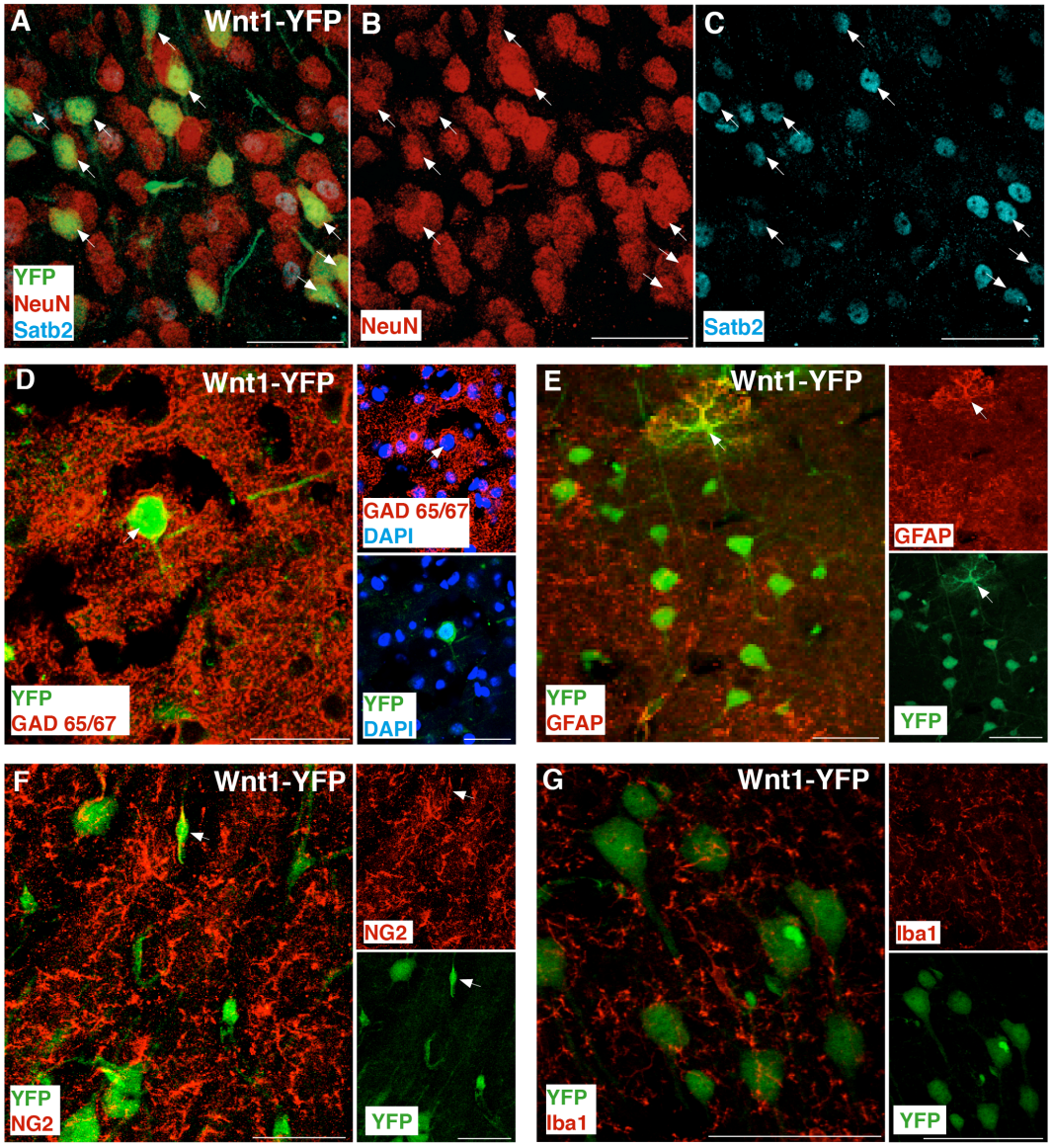

Figure 3.31. Quantification of markers expression in P21-30 Wnt1-YFP transgenic mice.

A-F, Brain section of a P21-30 Wnt1-YFP mouse, showing that YFP$^+$ cells in the cortex co-express the pan-neuronal marker NeuN (A,B, arrows). Among those, they express the neuronal marker Satb2 (A,C, arrows) and GAD65/67 (D, arrow). A minor proportion expressed the marker GFAP (E, arrow). The markers NG2 (E, arrow) and Iba1 (F) were minimally or not expressed. Scale bars: 50 µm.

3.6.2 – Tissue specific gene-fate mapping: Wnt1-Cre injected with LV-Brainbow 1.0(L)

To explore whether Wnt1-YFP$^+$ cortical neurons originated from meningeal-derived cells, we devised a tissue-specific gene fate mapping. We therefore injected a LV-Brainbow 1.0(L) in the meninges of P0 Wnt1-Cre mice (Figure 3.32A).

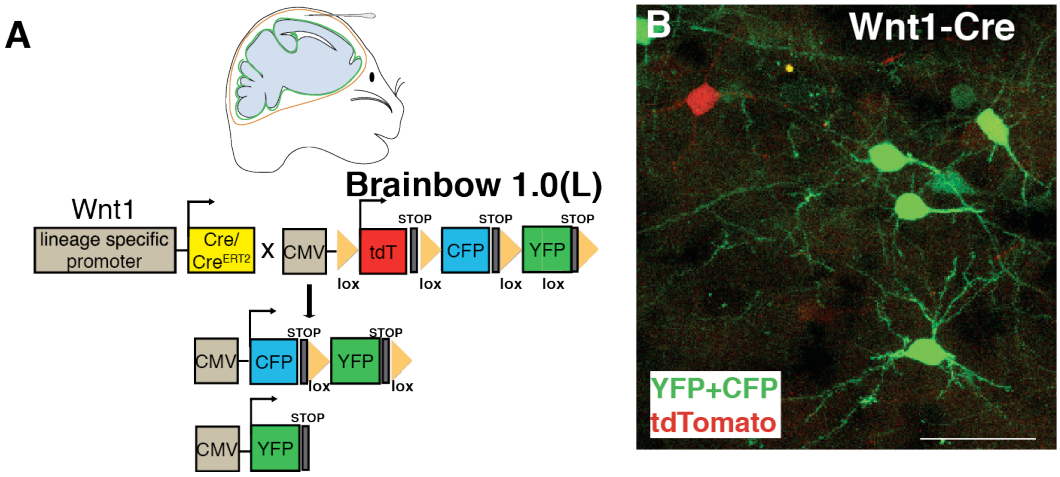

Figure 3.32. Recombination in Wnt1-Cre injected with the LV-Brainbow 1.0(L) at P0.

A, Schematic representation of the Cre induced recombination of the LV-Brainbow 1.0(L) injected in the meninges of lineage-specific Wnt1-Cre expressing mice. The red/brown and green line in the brain scheme denotes the outer layer (arachnoid) and inner layer (pia mater) of the meninges, respectively. The different colors after Cre-recombination are shown. CFP, cerulean fluorescent protein; tdT, tdTomato fluorescent protein; YFP, yellow fluorescent protein; CMV, cytomegalovirus promoter. **B,** Brain cortex of a P21-30 Wnt1-Cre mouse, injected at P0 with LV-Brainbow 1.0(L) in the meninges, stained for YFP/CFP and tdTomato, showing that the meningeal cells that migrated into the cortical layer I-IV were mostly Wnt1-Cre derived YFP^+/CFP^+ (green) cells. Scale bar: 50 µm.

Analysis of Wnt1-Cre mice, 21-30 days after LV transduction with the Brainbow 1.0(L) reporter in the meninges, revealed the presence of substantial numbers of CFP^+/YFP^+ cortical cells (70.2 % of all labelled cells) in the upper cortical layers I to IV (Figure 3.32B). Of those, 72.7% showed a typical neuronal morphology and expressed NeuN (Figure 3.33A-C,I). Another fraction of the CFP^+/YFP^+ cortical cells (13.9%) had a non-neuronal morphology and expressed GFAP (Figure 3.33F,I). These $GFAP^+$ cells were detected only by using the anti-GFAP polyclonal antibody (rabbit anti-GFAP, DAKO), while they could not be detected using the monoclonal antibody (mouse anti-GFAP, Sigma). $NG2^+$ and $Iba1^+$ cells were respectively, minimally or not detected amongst the CFP^+/YFP^+ cortical cells (Figure 3.33G-I). Among the CFP^+/YFP^+ neurons ($NeuN^+$), up to 66.0% expressed Satb2, while 6.7% expressed GAD65/67 (Figure 3.33D,E,I). These results suggested that a fraction of Wnt1-expressing meningeal cells generated cortical neurons in the adult brain.

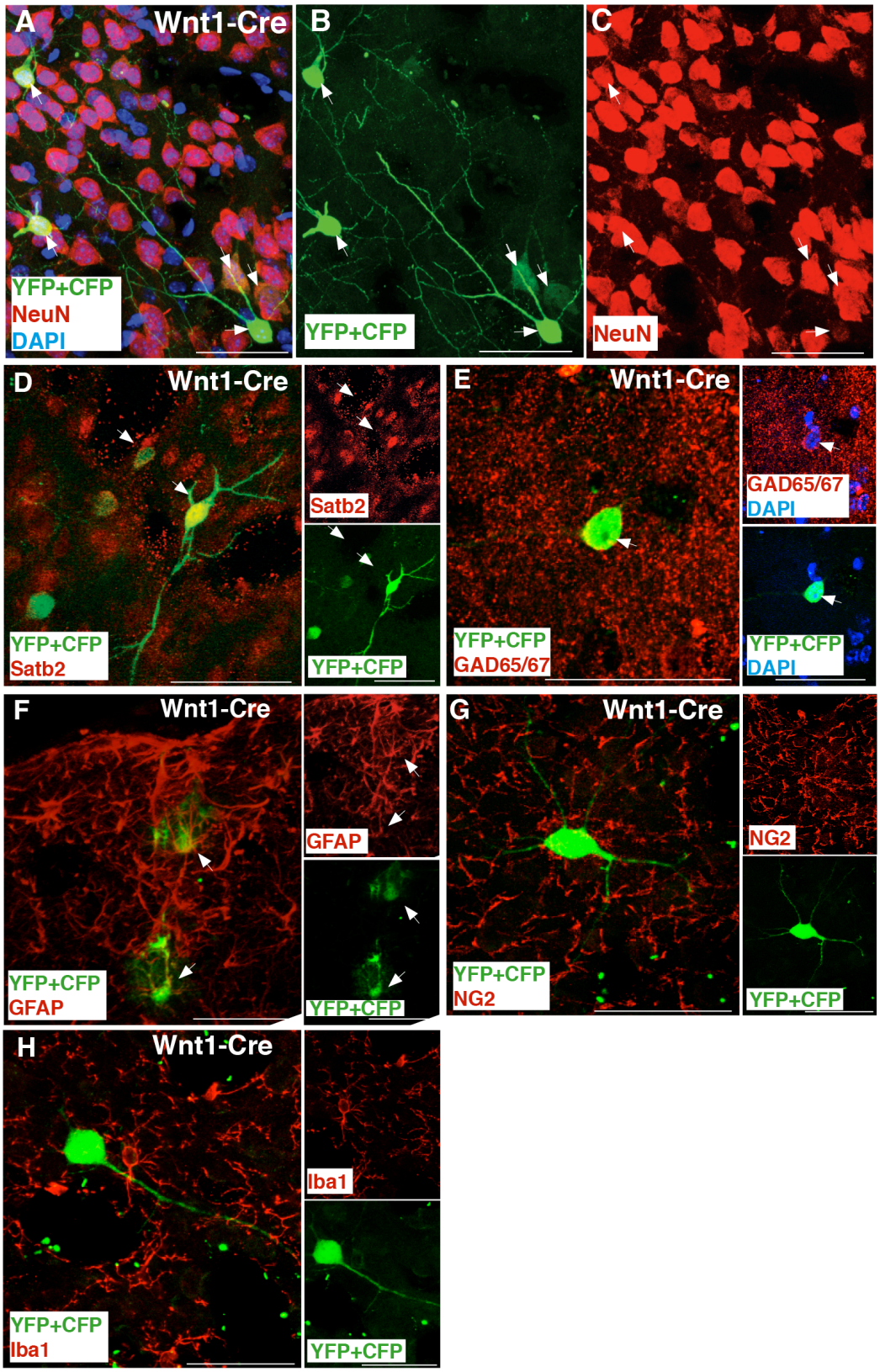

Figure 3.33. Quantification of markers expression in P21-30 Wnt1-Cre transgenic mice injected with LV-Brainbow 1.0(L) at P0.

A-H, Brain section of a P21-30 Wnt1-Cre mouse injected with LV-Brainbow 1.0(L) in the meninges, showing that YFP^+ cells in the cortex co-express the pan-neuronal marker NeuN (A-C, arrows). Among those, they express the neuronal marker Satb2 (D, arrows) and GAD65/67 (E, arrow). A minor proportion expressed the marker GFAP (F, arrow). The markers NG2 (G, arrow) and Iba1 (H) were minimally or not expressed. Scale bars: 50 μm.

Our results showed that meningeal cells give rise to cortical neurons in the postnatal mouse brain and, given their Wnt1 expression, are likely to have neural crest origin. It was already known that cephalic neural crest-derived cells can enter the forebrain early in development, where they differentiate mostly to $PDGFR\beta^+$ pericytes (Yamanishi et al., 2012). Here we show that these cells also migrate to the cortex to differentiate into neurons. Our finding that not all Brainbow-transduced cells in Wnt1-Cre mice give rise to CFP^+/ YFP^+ cortical neurons may suggest that meningeal cells with neurogenic potential have heterogeneous phenothype that may also include other still unknown population. Further studies using an inducible model (*e.g.* Sox10 line) are to be performed to confirm these initial results about neural crest derivation.

Control experiments of LV-Brainbow 1.0(L) recombination

To check the specificity of the LV-Braindow 1.0(L) and to explore whether only perivascular meningeal cells, but not vascular endothelial cells in the meninges gave rise to the cortical neurons, we used VE-cadherin(PAC)-Cre^{ERT2} mice, expressing a tamoxifen-inducible Cre^{ERT2} specifically in endothelial cells (Benedito et al., 2009). We transduced the meninges of P0 VE-cadherin(PAC)-Cre^{ERT2} pups with the LV-Brainbow 1.0(L), and injected them with tamoxifen, to induce recombination of Cre^+ cells. At P21-30, we only observed background numbers of CFP^+/YFP^+ neurons in the cortex (p=ns *vs* WT; $p<0.005$ *vs* PDGFRß-Cre) (Table 1), indicating indeed that perivascular (but not vascular endothelial) meningeal cells were a source of newly generated cortical neurons.

3.7 – Conclusions

Our results showed that embryonic-derived meningeal cells give rise to functional cortical neurons in the postnatal mouse brain. These meningeal neurogenic cells are distinct from and additive to the well-characterized neurogenic parenchymal radial glia. Moreover, we demonstrated that these cells belong to the perivascular PDGFRß$^{+}$ lineage.

Thus, PDGFRß$^{+}$ perivascular cells of the meninges and meningeal substructures migrate from their location outside the brain parenchyma to the cortex, to differentiate into neurons postnatally, and these neurons are functional under the electrophysiological point of view, integrated in the existing network and respond to pharmacological stimuli. Moreover, preliminary data suggested that this population could be embryonically derived from a subset of neural crest-derived population expressing Wnt1.

In Table1 are summarized all the quantifications of the phenotype characterization of labelled meningeal cells, analysed at P21-30, with indication of the lineages and the labelling technology adopted. Of note, the percentage of neurons derived from meningeal cells range from 75% to 82% of all labelled cells, demonstrating the high efficiency of neuronal differentiation of meningeal cells *in vivo*.

Mouse line	Gene transfer	Vector	Tamox	Cre^{+} cells	NeuN^{+} cells	GFAP^{+} cells	NG2^{+} cells	Iba1^{+} cells	Cells expressing neuronal markers	
									Satb2^{+}	GAD65/67^{+}
WT	LV	CherryRed	n.a.	n.a.	75.6 ± 1.4 (n=3)	0 ± 0 (n=4)	0 ± 0 (n=4)	0 ± 0 (n=4)	57.3 ± 2.1 (n=4) 75.8± 2.1*	15.5 ± 1.7 (n=4) 20.5± 1.7*
WT	LV	Brainbow 1.0(L)	n.a.	19.6 ± 2.4 (n=5)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
PDGFRβ-YFP	n.a.	n.a.	n.a.	n.a.	93.4± 2.3 (n=3)	0 ± 0 (n=3)	1.6 ± 0.7 (n=3)	0 ± 0 (n=3)	81.7± 3.7 (n=3) 87.6± 3.7*	3.4 ± 1.2 (n=3) 3.6± 1.2*
PDGFRβCre	LV	Brainbow 1.0(L)	n.a.	76.7 ± 1.6 (n=5)	82.2 ± 1.1 (n=4)	0.3 ± 0.3 (n=3)	1.0 ± 1.0 (n=4)	0 ± 0 (n=4)	70.7 ± 5.3 (n=5) 86.0 ± 5.3*	11.0 ± 1.1 (n=5) 13.4 ± 1.1*
VE-cadherin(PAC)-Cre^{ERT2}	LV	Brainbow 1.0(L)	P0	18.8 ± 2.7 (n=3) #	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Table 1: Quantitative analysis of the meningeal-derived cells in the cortex of P21-30 mice

Numbers are expressed as percentage of the total number of cells counted (for the WT line injected with the LV-CherryRed: CherryRed^{+} cells; for the -YFP mouse lines: YFP^{+} cells; for lines injected with the LV-Brainbow 1.0(L): CFP^{+}, YFP^{+} and dtT^{+} cells), or when indicated by an asterisk (*) as a percentage of the NeuN^{+}YFP^{+} cells (for -YFP mouse lines) or of the NeuN^{+}YFP^{+} and NeuN^{+}CFP^{+} cells (recombined LV-Brainbow 1.0(L) cells for the other mouse lines). For unknown reasons the LV-Brainbow 1.0(L) showed spontaneous excision of tdTomato and induction of CFP/YFP even without

the presence of Cre (this was observed also following cell transduction *in vitro*), as was the case in the meninges of Cre-negative mice (WT) or of mice, in which no contribution of meningeal cells to cortical cells was visualized (endothelial cell specific VE-cadherin(PAC)-Cre^{ERT2} mice). This background activation of the LV-Brainbow 1.0(L) reporter, assessed by counting the fraction of YFP^+ plus CFP^+ cells (% of YFP^+, CFP^+ and $tdTomato^+$ cells) was ~19%, thus much lower than the >70% values that we obtained in the mouse strains expressing active Cre. Data are expressed as mean ± SEM. n.a.: not applicable; Tamox: tamoxifen; NeuN: pan-neuronal - neuronal nuclei marker; GFAP: astrocyte marker glial fibrillary acidic protein; NG2: oligodendrocyte and pericyte marker neural/glial antigen 2; Iba1: microglia marker ionized calcium-binding adapter molecule 1; Satb2: neuron marker, special AT-rich sequence-binding protein 2; GAD65/67: GABA-ergic interneuron marker glutamic acid decarboxylase 65/67; EP: electroporation; LV: lentiviral transduction. #: p=ns *vs* WT and $p<0.005$ *vs* all other lines. Cre^+ cells: cells in which LV-Brainbow 1.0(L) reporter was recombined and yielded YFP^+/CFP^+ cells.

CHAPTER 4

DISCUSSION

The primary finding of this study is that embryonic-derived progenitors reside in meninges and give rise to functional cortical neurons in the postnatal mouse brain. We demonstrated that neurogenic meningeal cells migrate from their location outside the brain parenchyma, along the meningeal substructures, to the retrosplenial and visual motor cortices during the neonatal period. Subsequently, meningeal-derived cells differentiate into cortical neurons that are electrophysiologically functional, integrated in the existing network and responsive to pharmacological stimuli. In addition we found that these meningeal neurogenic cells belongs to the perivascular PDGFRß$^{+}$ lineage and are mainly additive to the well-characterized neurogenic parenchymal radial glia. Although the developmental origin of these cells still has to be elucidated, our preliminary data indicate a possible neural crest derivation.

To the best of our knowledge, this is the first study in which the role of meningeal cells in neurogenesis was investigated.

Based on the described results, we propose that meningeal cells, expressing PDGFRß and residing in the perivascular space, migrate along the meningeal substructures, underneath the hippocampus and through the choroid plexus, to the VZ. From the VZ, they proceed their migration to the lower and upper layers of the cortex, differentiating into functional cortical neurons. The proposed model is represented in Figure 4.1.

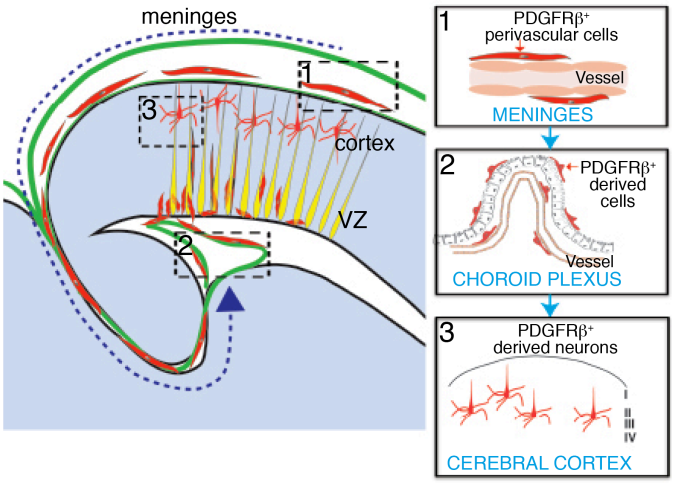

Figure 4.1. PDGFRß$^+$meningeal cells migrate into the cortex and differentiate into neurons. PDGFRß$^+$ cells reside in the meninges (1) and, postnatally, migrate along the meningeal substructures, underneath the hippocampus and along the choroid plexus (2), and then settle in the upper layers of the retrosplenial and visual-motor cortices to differentiate into functional neurons (3). Meninges: green; meningeal cells and derived neurons: red; radial glial cells: yellow. The path of migration is displayed by the blue dashed line.

The novelty and the outstanding relevance of our results reside in the fact that: i) they indicate that quiescent embryonic-derived neural progenitors may contribute to retrosplenial and visual motor cortex postnatal brain neurogenesis; ii) they broaden the concept of brain plasticity, since they highlight a novel mechanism of cell migration and plasticity inside the brain; iii) they challenge the dogma that only parenchymal cells can contribute to neurogenesis, and show that cells in the perivascular location can serve as a reservoir for neurogenesis.

4.1 - A new validated technique for meningeal cell labelling

The major bottleneck of this project was represented by the absence of a transgenic mouse model that labels exclusively meningeal cells. Therefore, as first step we developed a labelling technique that allowed us to specifically target meningeal cells, and not other structures in the brain.

We were able to specifically tag meningeal cells in P0 pups and track them during time, by means of injection of cell tracers in the meningeal subarachnoid space through a pressure-injecting microcapillary. To validate the injection technique, we tested different type of tracers: a lentiviral vector expressing fluorescent proteins

(LV-GFP or LV-CherryRed), a lipophilic dye (DiI), and a plasmid encoding for a red fluorescent protein (RFP-plasmid), transfected by electroporation. After the tracers injection, we extensively analysed all the brain sections, along the rostro-caudal and the medio-lateral axes, to confirm that the cell labelling was specific and confined to the meninges. Analysis of fluorescent expression at early time points after meningeal injection (6 to 15 hours later) revealed that the labelling was specifically confined in the meningeal tissue with no signal detected in other regions of the brain. We obtained the same results with all the tracers adopted, strongly consolidating our method.

As the lentiviral fluorescent expression is detectable only ~12 hours after the transduction, we further verified the specificity of the lentiviral transduction immediately (1 hour) after the injection. To this aim, we pursued two different strategies: i) we co-injected the lentivirus with a fluorescent dextran dye; ii) we injected CSFE-labelled lentiviral particles in the meninges. Analysis of all brain sections further confirmed that the viral diffusion immediately and few hours after the injection was confined to the meninges and meningeal substructures.

These data confirmed that we developed a consolidated method to specifically label and follow meningeal cells during the postnatal phase. On the other hand, this method presents some limitations: i) it does not allow detection meningeal cells during the embryonic development and ii) the efficiency of the cell labelling depends on the accuracy of the injection. The gold standard technique to perform population-specific fate map is in fact the use of inducible transgenic lines (Clarke and Tickle, 1999; Jensen and Dymecki, 2014; Joyner and Zervas, 2006). Further experiments will clarify the genotype of these meningeal cells and will set up the stage for the generation of a specific transgenic line for this population.

4.2 - Meningeal cells migrate to the cortex after birth

We observed that meningeal cells, during the postnatal period, are able to migrate from their location outside the brain parenchyma, to the upper layers of a specific region of the cortex. Meningeal cells migrated along the meningeal substructures that project underneath the hippocampus, to the VZ, the subplate and finally the cortical layers. We confirmed that this observation was not due to non-specific labelling caused by spreading of the LV particles in the CSF. In order to

exclude the unlikely event of targeting cells by direct injection of the LV in the lateral ventricle, we compared the fluorescent expression pattern obtained by the LV injection in the meninges and the LV injection in the lateral ventricle. The two completely different patterns of fluorescence expression and cellular labelling finally established that our method was specific for the meningeal compartment, excluding any targeting of the VZ during the injection.

It is already known that meningeal cells possess migratory potential following traumatic insults and injuries. Indeed, our group previously reported the ability of meningeal cells to proliferate and migrate in spinal cord parenchyma after spinal cord injury, where they contribute to the parenchymal reaction (Decimo et al., 2011). Another group, almost at the same time, described the migratory potential of meningeal cells in post-stroke area in brain (Nakagomi et al., 2012). However, meningeal cells migration from their location in physiological/ not injured conditions was never investigated before.

Indeed, in physiological conditions, despite the classical pathway of neuroblasts migration, from the SVZ to the OB radially along the RMS, and newborn granule cells migrating locally into the DG, migrating cells in the postnatal cortex are poorly observed. However, migration of NPs from the VZ-SVZ to the cortex was reported, in physiological condition and in postnatal brain (Zgraggen et al., 2012). This work supports the idea that, albeit in rare cases, migration of NPs into the cortex in physiological condition is a plausible event.

We showed that meningeal NPs migrate postnatally along meningeal substructures to the VZ, and then to the upper layers of the cortex. Although the transition from VZ-SVZ to the cortical layers may resemble the migratory pathway and direction already described for postnatal migration of NPs (Zgraggen et al., 2012), it is still not clear what is the signalling driving meningeal cells along the meningeal substructures and then to the cortex.

Migration of neuroblasts and NSCs in the postnatal and adult brain has been shown to require the expression of different molecules, including the morphogen sonic hedgehog (Shh). Indeed, migrating neuroblasts in the SVZ and RMS have been described to express Patched, the Shh receptor, in response to the presence of Shh in

the CSF and in SVZ (Angot et al., 2008). Moreover, deletion of the Shh signalling impairs the migration of neuroblasts postnatally (Balordi and Fishell, 2007).

Another important protein involved in NSCs/ NPs migration is reelin (D'Arcangelo, 2014; Li et al., 2009; Sekine et al., 2014). Indeed, reelin not only regulates neuronal positioning in cortical layers during the embryonic development, up to their final positioning beneath the MZ; it is also involved in the termination of neuronal migration by degrading Dab1 via the SOCS7- Cullin5- Rbx2 system (Sekine et al., 2014).

Together with these pathways, an important contribution on NSCs/ NPs migration is given by stromal derived factor-1 (SDF-1, also known as CXCL12), a potent chemokine produced by the meninges and regulating neuronal homing processes during development, through the binding to its receptor CXCR4 (Borrell and Marín, 2006; Klein et al., 2001; López-Bendito et al., 2008). SDF-1 regulates the migration and positioning of neurons into the embryonic cortex (Kokovay et al., 2010b); it has also been shown to promote post-stroke neuronal migration and behavioural recovery (Ohab et al., 2006). Taken together, these data suggest an important role for the SDF-1-CXCR4 axis in NPs migration.

Further investigations on the expression of these regulators and their role in meningeal cell migration are needed to highlight the mechanisms underlying meningeal NPs migration, and to finally define the precise path of migration along the substructures. However, due to the presence in meninges of the SDF-1-CXCR4 axis and the importance of this signalling for NPs migration in development, in postnatal physiological and in pathological conditions (Bifari et al., 2015; Decimo et al., 2011; Kokovay et al., 2010b; Li et al., 2009; Ohab et al., 2006), we speculate that this mechanism could be the best candidate in guiding the migration of NPs from the meningeal tissue to their final position in the cortex; in addition, this pathway could be supported by reelin signalling from the subpial/ MZ.

By means of time-lapse imaging experiments, we observed that meningeal cell migration occurs by locomotion, through a continuous saltatory activity from the lower to the upper layers of the cortex.

The "locomotion" migration refers to freely migrating immature neurons or neuroblasts that migrate with a saltatory motion (Rubenstein and Rakic, 2013a). This

migration modality was first described by Pasko Rakic for neuroblast migration to the upper layers of the cortex in monkeys (Rakic, 1972; Tabata and Nakajima, 2003; Nadarajah et al., 2003). In the postanal period, migration by locomotion is the principal migratory mode, as no other modalities (either somal translocation and multipolar migration, that were described to occur during early embryonic corticogenesis) were observed (Miyata et al., 2001; Zgraggen et al., 2012).

We measured the velocity of meningeal migrating cells and detected an average speed of ~50 μm/hour. This velocity is in line with the range of speeds observed for NPs, that has been described to vary from 10 to >60 μm/hour. Indeed, while some NPs have been observed to migrate radially at 10-15 μm/hour (Babona-Pilipos et al., 2011; O'Rourke et al., 1992), higher velocity have been reported, such as ~30-50 μm/hour (Bolteus and Bordey, 2004; Nadarajah et al., 2003; Pramparo et al., 2010), or even faster (>60 μm/hour, Nadarajah et al., 2001; Youn et al., 2009). The meaning of these discrepancies has not been elucidated yet.

Meningeal cells display a modality of migration resembling that of NPs migrating from the VZ to the cortical layers, both in terms of the locomotor-saltatory movements and reported velocity. However, no migration of meningeal cells from their original location to other regions has ever been described in physiological conditions, highlighting the importance of our observations. Still, a detailed and more accurate investigation of the migratory behaviour of meningeal cells is needed. It would be interesting to explore how meningeal cells migrate through the meningeal substructures and the layers of the postnatal cortex, and whether they are RG-guided or move independently from that scaffold. Several experiments are needed to uncover the signalling and molecules involved in this migration. A detailed investigation on the path that meningeal-derived cells follow could be provided by two-photon microscopy, which will allows higher resolution of analysis in living animals.

4.3 - Migrating meningeal cells are originated in the embryonic period

Our results showed that migrating meningeal cells, labelled with LV-Cherry at P0 and analysed at P21-30, are originated embryonically. Indeed, birthdating experiments with single injection of EdU at precise time points during embryonic

development, demonstrated that an important fraction of CherryRed^{+} cells had incorporated EdU between E13.5 and E16.5, with a peak of incorporation at E14.5. These cells then maintain their quiescent state until the postnatal period, when they migrate to the cortex and differentiate into neurons.

The description of NPs retaining quiescence after the proliferative phase was already reported in several works, which described the presence of quiescent progenitors in the adult brain. Indeed, recent studies demonstrated that a subset of ventricular RGCs, generated between E13.5 and E15.5, becomes quiescent and acts as NSCs only later, in the adult mouse brain (Fuentealba et al., 2015; Gage and Temple, 2013). Moreover, evidences in zebrafish suggested that new neurons can be generated not only by amplification of the neuronal output by intermediate progenitors, but also by direct conversions of stem cells into postmitotic neurons following brain injury, in absence of a proliferating step (Barbosa et al., 2015). These studies demonstrated new different mechanisms of neuronal production in the postnatal cortex, both by embryonic derivation, quiescence and postnatal reactivation, and by direct conversion of NPs into postmitotic neurons.

Postnatal differentiation and migration from the VZ was observed by the group of Jozsef Kiss (Zgraggen et al., 2012). They showed that new neurons are added in the medial limbic cortex of postnatal rats and that these neurons are generated around E18-E19 and migrate only postnatally from the VZ to the cortex, differentiating into projection neurons. These results were in accord to what observed by the group of Hidenori Tabata (Tabata et al., 2009), which demonstrated that NPs stay quiescent in the VZ several hours before becoming postmitotic and accumulate in the SVZ only later. These works again support the idea that NPs may retain quiescence in the VZ and migrate and differentiate into neurons later in development or postnatally, due to their embryonic commitment. Overall, all these studies support the mechanism of postnatal neuronal differentiation of embryonic-generated meningeal-derived cells.

Moreover, in this time window (from E13.5 to E16.5) neurons of the subcortical and callosal projecting neurons of layer V, pyramidal neurons of the layer IV, granular neurons and callosal projecting neurons of the upper layers are generated (Greig et al., 2013; Molyneaux et al., 2007). These data reflect the time of origin and fate commitment of meningeal-derived neurons. Together with our results, this

suggests that cells in the meninges, generated at E13.5-E16.5, retain quiescence until the postnatal period, migrate to specific cortical layers, and there they differentiate, in accord to the commitment they received at their time of origin.

Our results, showing that NPs residing in the meninges differentiate only postnatally, support the idea of the existence of NPs-pools in the postnatal brain. These cells are generated embryonically and maintained as reservoir until birth, then migrate and differentiate to support (or complete) cortical development in the postnatal period.

4.4 - Meninges contribute to postnatal neurogenesis

We demonstrated, for the first time, that the newly described NP population in the meningeal tissue is neurogenic *in vivo*, in the postnatal period. Indeed, quiescent progenitors generated in the embryonic period, around E13.5-16.5, showed a "reactivation" at P0, with migratory activity and differentiation into the cerebral cortex.

Postnatal neurogenesis occurs only in two neurogenic regions: SVZ and SGZ, and newly formed neuroblasts migrate locally to the DG of the hippocampus and to the OB through the RMS, where they continually replace local interneurons (Imayoshi et al., 2008). However, several new evidences have been reported regarding neurogenesis in the adult striatum, amygdala, hypothalamus, substantia nigra and brainstem (Gould, 2007).

The case of adult neurogenesis in the cortex is controversial: while different groups described generation of new neurons in the postnatal cortex (Menezes et al., 1995; Gould, 2007; Costa et al., 2007), others state that corticogenesis is embryonically restricted and may proceed up to the perinatal period, but not further (Bhardwaj et al., 2006).

However, while the proliferation of new neurons may indisputably be restricted only to the embryonic period, these newborn cells may remain quiescent until birth. Our results are supported by recent works, already mentioned, demonstrating the presence of NPs that remain quiescent until the postnatal period (Tabata et al., 2009; Fuentealba et al., 2015), and the mechanism of direct conversion of stem cells into post-mitotic neurons in zebrafish (Barbosa et al., 2015). Although no newborn

neurons are generated and added in the cortex postnatally (Fuentealba et al., 2015; Gage and Temple, 2013; Kriegstein and Alvarez-Buylla, 2009), and virtually addition of new proliferating neurons after birth is absent (Altman, 1969; Rakic, 2002), these newly described mechanisms support our results about addition of quiescent meningeal-derived neurons in the postnatal cortex.

Moreover, a 6.4-fold increase in the net brain mass has been observed in the first 3 months after birth. The postnatal increase in neuronal numbers seems to follow an oscillatory pattern, with a rapid growth in neuronal number at P3-P7 followed by a decrease until P15. Non-neuronal cell types increase in number from P7, once neuronal addition is mainly over in the cortex, hippocampus and OB (Bandeira et al., 2009). These patterns of neuronal addition to the cortical tissue suggest a time-window where migratory activity of immature postnatal neurons could occur.

Overall, there are controversial evidences about the addition of new neurons in the postnatal cortex. Our results are supported by the mechanism of direct conversion and by new evidences about NPs quiescence. The net addition of cells in the postnatal cortex suggests that the migratory activity of embryonically generated neurons continues after birth, and our data about meningeal cell migration and generation of cortical neurons suggest that meninges could actively contribute to this event.

4.5 - Meningeal cells differentiate in functional cortical neurons

By labelling meningeal cells at P0 with different techniques (*i.e.* LV transduction and plasmid electroporation) and analysing the phenotype at P21-30, we were able to identify a remarkable population of labelled neurons, implicating that the majority of meningeal-derived cells have neurogenic potential *in vivo*. The neurogenic potential of both brain and spinal cord meninges was already described *in vitro* and after transplantation *in vivo* (Bifari et al., 2009; Decimo et al., 2011; Nakagomi et al., 2011; Nakayama et al., 2010), but the contribution to *in vivo* neurogenesis in physiological conditions was not investigated yet.

Characterizing the meningeal derived CherryRed$^+$ neurons, we found an interesting proportion between excitatory and inhibitory neurons. Indeed, among the ~75% of NeuN$^+$ cells, 76% were Satb2$^+$, a marker which labels cortical layer I-IV

neurons in the cortex (Huang et al., 2013), while 20% expressed the inhibitory marker GAD65/67. These percentages are highly representative of the endogenous neuronal population in the adult brain cortex (Gonchar et al., 2007; Liu et al., 2010; Markram et al., 2004; Ozeki et al., 2009; Priebe and Ferster, 2008; Sillito, 1975), suggesting that, when the meningeal population is postnatally added to the existing cortical neuron population, the electrophysiological balance in the existing network is maintained.

Moreover, electrophysiological analysis comparing the functionality and the properties of CherryRed$^+$ neurons and neighbouring cells revealed that most CherryRed$^+$ neurons acquired an excitable electrical phenotype similar to that of established cortical neurons and largely reminiscent of fast-spiking neurons, as opposed to regularly firing (Markram et al., 2004) and mature neurons (Espósito et al., 2005; Mongiat and Schinder, 2011; Mongiat et al., 2009). Interestingly, 1 out of 12 recorded cells displayed typical interneuron-like properties, strongly confirming the proportion of neuronal population obtained by immunohistochemistry. Importantly, CherryRed$^+$ cells were functionally integrated in cortical local synaptic networks, both in excitatory and inhibitory local microcircuits.

The biological function of postnatal meningeal-derived neurons remain unknown; however, we speculate that they may contribute to information processing, in a different way compared to mature neurons in the same circuit, as hypothesized for NSCs adult-born neurons. In addition, they may promote plasticity of existing circuitry by making new synaptic contacts with mature neurons (Ming and Song, 2011).

Meningeal-derived cells mostly differentiate into NeuN-expressing neurons, and this high efficiency in neuronal differentiation was observed using different labelling techniques (LV-injection, plasmid electroporation), and both in wild-type and transgenic animals (Rosa-YFP, Wnt1-Cre, PDGFRß-Cre). This observation strengthens our previous results demonstrating that rodent meninges host a population endowed with neuronal differentiation throughout different developmental stages (Bifari et al., 2009, 2015; Decimo et al., 2011; Nakagomi et al.,

2011, 2015b; Nakayama et al., 2010), and present all the features of a classical NSC niche (Bifari et al., 2015).

4.6 - Meningeal-derived neurons are localized in the retrosplenial and visual-motor cortices

Our results demonstrated that, in postnatal brain, a subset of NPs present in the meninges migrates to specific regions of the cortex, the retrosplenial (RSC) and visual motor cortices (VMC, or secondary visual cortex) and differentiate into functional neurons. Previous observations that neurons from these regions are generated at E14.5-E15.5 (Deng and Elberger, 2001) reflect the time of generation of meningeal-derived neurons, again supporting their embryonic derivation.

The RSC is part of the posterior cingulate cortex (PCC, Wenk, 2003) and has been shown to be involved in spatial memory and navigation (Aggleton and Vann, 2004; Vann and Aggleton, 2004; Vann et al., 2009). Studies involving patients suffering from Alzheimer's disease (AD) described cellular atrophy (Pengas et al., 2010; Scahill et al., 2002) and hypometabolism (Desgranges et al., 2002) in the PCC and RSC, indicating that these regions are vulnerable to neurodegeneration during the progression of AD and that dysfunctions in these regions are likely to contribute to cognitive defects.

Neurons located in the VMC, mostly referred as secondary visual cortex (Gazzaniga et al., 2002), are tuned to simple properties such as orientation, spatial frequency and color. It has been shown that dysfunction in the visual cortex, either primary or secondary, is related to visual impairment in AD, indicating involvement of the VMC in the pathological changes occurring during the progression of AD (Mielke et al., 1995).

We speculate that meningeal-derived neurons, located in RSC and VMC, could be involved in information processing for spatial and navigation memory and that they may be implicated in the region-specific neurodegeneration observed in cognitive disorders as AD. Therefore, it would be interestingly to study pharmacological modulation of meningeal-derived neurons, as this can exploit a therapeutic effect in AD.

4.7 - Meningeal-derived neurons belong to the PDGFRß population

To the best of our knowledge, there is no transgenic model that exclusively labels meningeal cells. Although our labelling method provided interesting results about meningeal cell fate, the use of transgenic models is still the gold standard to perform lineage tracing. Thus, in order to investigate if meningeal-derived neurons were generated from specific lineages, *i.e.* the RG lineages, we employed two different transgenic lines: the GLAST-YFP and the Nestin-YFP lines. Lineage tracing experiments revealed that only a small fraction of meningeal-derived CherryRed^{+} neurons are derived from the radial glial populations GLAST or Nestin. Induction of the activation of GLAST and Nestin-promoters at E13.5, a time when most of the RG-neurogenic cells are produced (Götz and Barde, 2005; Hartfuss et al., 2001; Lendahl et al., 1990; Malatesta et al., 2000) demonstrated that meningeal-derived neurons are a separate population from the classical RGCs, although a small population of GLAST^{+} or Nestin^{+} cells may contribute to meningeal-derived neurons. Nevertheless, the debate about cortical neurogenesis in physiological conditions, especially for those niches that are not RG-derived, is still intense.

We demonstrated that a subset of meningeal-derived neurons have a perivascular identity and they belong to the PDGFRß $^{+}$ population of the meninges. The neurogenic potential of perivascular cells was already observed both *in vitro* (Nakagomi et al., 2015b, 2015c) and in response to injury *in vivo* (Nakagomi et al., 2011). Thus, the putative neurogenic role *in vivo* in physiological condition was an intriguing field to investigate. Moreover, the link between the vascular and the neurogenic niches was extensively reported (Jenny et al., 2009; Ottone et al., 2014; Palmer et al., 2000), and pericytes not only displayed multipotency potential *in vitro* (Dore-Duffy et al., 2006; Zimmerlin et al., 2012), but also have been driven to reprogram into neurons in culture (Karow et al., 2012b). Furthermore, a recent study demonstrated that the PDGFRß signalling: i) is required for RG cell cycle progression in developing human neocortex; ii) is expressed in the VZ of lateral mouse cortex and iii) is sufficient to promote RG identity (Lui et al., 2014). Taken together, these reports define an important role for PDGFRß expression in neuronal development and in traumatic response.

We analysed PDGFRß expression in the cortex of PDGFRß-YFP mice. In line with reports that show that PDGFRß is also expressed by a subset of neuronal progenitors in the VZ of newborn pups (Ishii et al., 2008b; Lui et al., 2014; Williams et al., 1997), but contrary to what described about the weak or absent expression of PDGFRß in the adult mouse brain neurons (Smits et al., 1991; Winkler et al., 2010), we found that the majority of the YFP^+ cells in the adult brain cortex differentiate into $NeuN^+$ neurons. Thus, at the best of our knowledge, this is the first project reporting the neurogenic potential of an adult perivascular population *in vivo*.

However, since other cell types in the brain also expressed PDGFRß, this experiment did not exclude the possibility that PDGFRß-YFP^+ cortical neurons originated from other PDGFRß-YFP^+ cells in non-meningeal brain regions. We therefore devised a tissue-specific gene fate mapping technique, that specifically labelled only meningeal-derived PDGFRß cells. The results obtained with this experiment confirmed that perivascular PDGFRß cells in the meninges differentiate with high efficiency into neurons.

This was the first description of a specific perivascular population, not derived from RG population, with high neurogenic potential *in vivo* and in physiological conditions, strongly confirming the relationship between the (peri)vascular and neurogenic niches.

4.8 - Preliminary results suggest a neural crest developmental origin of meningeal-derived neurons

In light of several considerations, we investigated the contribution of the neural crest- Wnt1 population to postnatal neurogenesis. First, forebrain meninges are neural crest-derived, in contrast to mid- and hindbrain meninges that are mesoderm-derived (Couly and Le Douarin, 1987; Jiang et al., 2002; Siegenthaler and Pleasure, 2011a). Second, previous report showed efficient labelling of forebrain meninges and meningeal brain projections using a Wnt1-Cre reporter line (Jiang et al., 2002). Therefore, we decided to adopt this specific transgenic line to identify the developmental origin of meningeal-derived neurons.

Our preliminary investigation, by lineage tracing experiments and tissue-specific gene fate mapping, showed that a subset Wnt1 population of the meninges contribute to the neuronal addition observed in the postnatal period. These results, however, are controversial to previous reports, that stated that the neural crest gives

rise only to neurons and ganglia of the PNS, and no CNS neurons are neural crest-derived (Rubenstein and Rakic, 2013b). Nevertheless, meningeal-derived neurons are generated in the same time window of neural crest cells (around E10.5-E13.5, Jiang et al., 2002; McLone and Bondareff, 1975), leading us to speculate that meningeal-derived neurons may be neural crest-derived. Moreover, the presence of YFP^+ neurons in the Wnt1-YFP adult, and the neuronal differentiation of recombined Wnt1-Cre meningeal cells transfected with LV-Brainbow 1.0(L), gave us more clues about the neural crest-derivation of those neurons.

These preliminary results need to be confirmed by further investigations, using an inducible transgenic line specific for the neural crest lineage.

4.9 - Final conclusions and translational relevance

We found that $PDGFR\beta^+$-derived meningeal cells can migrate and differentiate into functional cortical neurons in the postnatal mouse brain. Our results are relevant for multiple reasons: i) they indicate that quiescent embryonic-derived neural progenitors may contribute to postnatal brain neurogenesis; ii) they broaden the concept of brain plasticity; and iii) they challenge the dogma that only parenchymal cells can contribute to neurogenesis, highlighting the importance of perivascular cells as a reservoir of neurogenic cells.

These results may have a great impact in the neural stem cell-field, as no addition of endogenous neurons has ever been described in the postnatal and adult cortex. Due to the absence of neuronal replacement after degeneration and cellular loss, the cerebral cortex is one of the brain areas more affected by neurodegenerative diseases. In spite of the presence of a primary *noxa patogena*, which elicits an endogenous response in the CNS aimed at regenerating the damaged tissue, this reaction is insufficient to restore neuronal function and/or to block disease progression. Endogenous neurogenic response of NSCs to diseases has been shown, also in humans (Jin et al., 2006; Minger et al., 2007; Nogueira et al., 2014); however, adult NSCs of the SVZ generate mainly glia cells in response to pathological conditions (Grégoire et al., 2015; Merkle and Alvarez-Buylla, 2006). Until now, endogenous stem cells able to generate new neurons in the postnatal cortex have never been identified. In this context, our findings that a novel population generates

new cortical neurons in the postnatal period may represent a completely new opportunity for the treatment of neurodegenerative diseases.

Indeed, meningeal cells may have a physiological role as endogenous stem cell reservoir, that could be exploited in regenerative medicine for neurological and neurodegenerative diseases. These findings may be in fact translated to pathological/injury models and they may lead to therapeutic strategies and to the identification of a possible cell-based therapy approach neurodegenerative disorders. Two possible applications of meningeal-derived cells in neuroregenerative medicine are represented by pharmacological modulation of the endogenous response or autologous transplant of meningeal cells.

PHARMACOLOGICAL MODULATION

Pharmacological modulation of NPCs has already been shown to represent a therapeutic alternative to cells transplantation in neurodegenerative diseases treatment (Androutsellis-Theotokis et al., 2009).

Evidence of exogenously transplanted cells functionally integrating into the host brain, replacing cells, and having a behavioural benefit have been shown in different disease models, along with the ability of some cell sources to stimulate endogenous neuroprotective and restorative events through a bystander effect (Trueman et al., 2013). On the other hand, several approaches to stimulate endogenous neurogenesis have been tested but still no significant results have been obtained (Fallon et al., 2000; Munoz et al., 2005; Palfi et al., 2002).

Until now, endogenous stem cells able to generate new neurons in the postnatal cortex have never been identified. In this context, our findings that a novel population generates cortical neurons postnatally may represent a completely new opportunity for the treatment of neurodegenerative diseases. The discovery of a cell population contributing to neurogenesis in specific cortical areas (RSC and VMC), regions that have been shown to be vulnerable to neurodegeneration in AD, may be a novel approach to investigate a novel therapy, basing on the endogenous role of the meningeal-derived neuronal population. However, further investigations are needed to first identify the function and physiological role of meningeal-derived neurons in RSC and VMC.

In order to explore a possible endogenous response of PDGFRß$^{+}$ meningeal cells to AD and, in general, to neurodegenerative diseases and injuries, an intensive study about the mechanism underlying PDGFRß$^{+}$ meningeal cells migration and proliferation, and the molecules involved in those mechanisms, is essential to develop an efficient pharmacological therapy to stimulate those cells in a response of a neuronal degeneration.

TRANSPLANTATION

Soon after the *in vivo* identification of stem cells from the CNS, different procedures have been developed to obtain a large number of NPCs *in vitro*, for transplantation purposes (Martino and Pluchino, 2006).

Transplantation of adult SVZ-derived NPCs has been demonstrated to have a therapeutic effect in ischemic stroke lesions (Bühnemann et al., 2006), showing neuronal and astrocyte differentiation, migration to lesioned parenchyma and integration in host circuits. However, the beneficial effect of cell transplantation is mostly due to a bystander effect of grafted cells, with a minor role played by direct cell replacement. Indeed, in a mouse model for multiple sclerosis (MS) called experimental autoimmune encephalomyelitis (EAE), the transplantation of adult NPs displayed an improved outcome in locomotor function, mainly to immune-like functions that promote long-lasting neuroprotection (Pluchino et al., 2005). NPs expanded from the adult rat SEZ were grafted to the striatum of adult rats with 6-OHDA-induced brain lesions, in order to investigate the effect of transplantation in Parkinson's like model. Although no cellular differentiation has been described, beneficial effect was reported, probably thanks to a graft's action on the maintaining on the progenitor niche (Richardson et al., 2005). The efficacy on adult NPs was tested also on an acute model of SCI. Transplantation resulted in positive effects, including increased amounts of myelin in the injured area, recovery of hindlimb locomotor function and hindlimb sensory responses, as determined by functional magnetic resonance imaging. However, aberrant axonal sprouting associated with hypersensitivity of forepaws was described, suggesting that transplantation can cause severe side effects and call for caution in the consideration of clinical trials (Hofstetter et al., 2005).

Collectively, these results suggest that transplantation may exhert a therapeutic effect in several disease models, not only due to a direct differentiation and replacement in the damaged area, but also thanks to a bystander effect. As mentioned above, several attempts for NPC-based therapies for CNS disorders and injuries have been developed; however, there are still important issues that need to be resolved before any potential applications of such promising therapies in humans can be foreseen. Not only the ideal cell source for transplantation (embryonic *versus* adult), but also the best route for cell administration (local *versus* systemic) have to be determined (Martino and Pluchino, 2006).

The isolation of PDGFRß$^+$ cells from adult meninges and the investigation of the effect of the engrafting of these cells in a disease model may help us in understanding the neuroregenerative-protective properties of meningeal-derived PDGFRß$^+$ neurons (Karow et al., 2012b; Nakagomi et al., 2015c). The high accessibility of the meningeal tissue compared to other neurogenic regions, the less ethical concerns about the use of adult cells compared to embryonic-derived, and the high efficiency of neuronal differentiation *in vivo*, without any need of reprogramming, make meningeal cells highly attractive to extraction and grafting in an autologous setting. However, despite all these intriguing properties, cell-based therapies raised and are still raising important ethical, technical and immunological concerns (Li et al., 2008).

Overall, our data demonstrated that meninges, residing outside the brain parenchyma, harbour cells endowed with neuronal differentiation potential *in vivo*. The location of this tissue, easily accessible for sampling without impairing or damaging cortical structure and function, and the high *in vivo* differentiation potential suggests the application of PDGFRß$^+$ meningeal cells for autologous sampling. In addition, another application and final goal could be to provide novel pharmacological targets to modulate the endogenous response of PDGFRß$^+$-derived meningeal cells, opening novel studies and encouraging perspectives in brain repair following neuronal degeneration.

The modulation of this neurogenic population could be a novel therapeutic strategy to boost endogenous regeneration in neurodegenerative diseases. This may shift the paradigm of brain plasticity from a timely and tightly restricted area where

stemness is confined, to a potential "ready to use" widespread NSC potential located in the perivascular space.

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